EFFECTS OF COPPER ON GILL STRUCTURE AND PHYSIOLOGY IN *CARCINUS MAENAS*

by

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ABSTRACT

The effects of sublethal copper exposure at three levels of biological organisation were studied in the common shore crab *Carcinus maenas* (L.) (Crustacea, Decapoda). The three levels included the ultrastructure of respiratory and osmoregulatory gill tissues; ventilatory physiology (scaphognathite activity); and tissue metallothionein levels.

Respiratory gill epithelia were more sensitive to sublethal copper exposure than osmoregulatory gill tissues. The cellular damage observed included severe epithelial necrosis and vacuolation, hyperplasia and haemocyte infiltration. In the respiratory gills, these changes were first present following exposure to 100 μg Cu L^{-1}. At 500 μg Cu L^{-1}, there was complete degeneration of the epithelia. In osmoregulatory gills, lipofuscin granules were formed at 300 μg Cu L^{-1}. Signs of cellular damage (as observed in respiratory gills) appeared in the osmoregulatory gills only following exposure to 500 μg Cu L^{-1}, and were restricted to areas proximal to the marginal canal. Copper concentrations below 100 μg Cu L^{-1} had no effect on gill tissues. This result is discussed with reference to previous studies, and related to inter-population differences and exposure techniques. Gill ultrastructural differences were observed in crabs from two estuaries with different levels of water-borne trace metals, and in crabs transplanted from the cleaner to the more polluted site. Differences included varying densities of plasmalemmal folds and frequencies of cellular vacuolation, as well as composition and thickness of algal surface layers on the gill cuticle.

Following laboratory copper exposures (500 μg Cu L^{-1}), gill ultrastructural "damage" and tissue metallothionein levels were related to changes in scaphognathite activity. Physiological effects, including changes in scaphognathite rate and periods of apnoea, were exacerbated by increased temperature and hypoxia. Changes in scaphognathite activity and metallothionein levels were not consistent following several exposures to the same level of copper; results are discussed in relation to physiological influences. In contrast, gill ultrastructure showed consistent deterioration following exposure to 500 μg Cu L^{-1}. Gill ultrastructure represents a reliable indicator of exposure to copper at this concentration compared to both scaphognathite activity and metallothionein concentrations.

When the war between the frogs and the mice was going in favour of the mice, and it seemed that the frogs would be defeated, Zeus sent help to the frogs:

"There came suddenly the shield backs,
the hook lips,
the side walkers,
the twisted,
the nipper-mouthed,
bone-skinned,
bone-like,
broad-backed,
with gleaming shoulders,
the bowed-legged,
the tight-lipped,
seeing from their chests,
eight-footed,
double-headed,
handless,

who are called the crabs,
who struck the mice on their tails
and their feet and their hands with their mouths-
and thus put the mice to flight."

[From *"Batrachomyomachia"* ("Battle of the frogs and the mice")

Homer, Opera, Vol. 5, Oxford Uni. Press]

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LIST OF ABBREVIATIONS

A	apical membrane/plasmalemma
AAS	atomic absorption spectroscopy
AF	afferent vessel
AL	algal/bacterial layer
ANOVA	analysis of variance
ant	anterior
apn	apnoea
ATP	adenylate tri-phosphate
AWP	apparent water permeability
B	basal membrane
bpm	beats per minute
BR	branchiostegite
C	control
CA	cardiac activity
CAPMON	computer-aided physiological monitor
CE	cristae
CH	chief cell
Co	collar
CP	haemolymph capillary
CR	central raphe
Ct	computer-transducer interface
CU	cuticle
CV	central vacuole
D	detrital layer
Dc	distribution chamber
DG	distal gill region
Di	perforated dividing walls
DN	maximum distance between frequency plots
DTNB	dithio-bis/2-nitrobenzoic acid
DTT	dithiothreiotol
DW	dry weight
E	efferent vessel

EDTA	tris-ethylenediaminetetraacetic acid
EP	epithelium
Ex	exposed
EZY	enzyme activity
Fi	flow inducer
G	gills
GL	glycogen
GLM	general linear models
GO	golgi body
GR	granule
GSH	glutathione
H	hypoxia
HA	haemocoel (haemolymph space)
HC	haemocyte
HCY	haemocyanin concentration
He	heart
If	in-flow
IS	intralamellar septum
K-S	Kolmogorov-Smirnov
L	left scaphognathite
LY	lysosome
LF	lipofuscin granule
LSD	least significant difference
M	marginal canal
MF	microfilaments
MG	mid gill region
mgg	midgut gland
MI	mitochondria
M (O_2)	oxygen consumption
MT	metallothionein
Mx	seawater and stock solution mixing cylinder
N	normoxia
NU	nucleus
NE	nephrocyte

NS	not significant
Of	out-flow
P	pedicels
PaO_2	arterial haemolymph partial oxygen pressure
PC	personal computer
PG	proximal gill region
PI	pillar cell
PMSF	phenylmethylsulphonylfluoride
post	posterior
PT	pressure transducer
Q	perfusion
R	right scaphognathite
rev	reversal
RSE	relative standard error
S	satellite vacuoles
Sc	scaphognathite
Se	infra-red sensors
Sf	seawater flow
SFG	scope for growth
TEM	transmission electron microscopy
V	ventilation
VA	vacuole
V/Q	ventilation/perfusion ratio
$V_{V/mit}$	mitochondrial area volume
WW	wet weight

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CHAPTER 1

GENERAL INTRODUCTION

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1.1 The Need for an Integrated Approach to Contaminant Effects

Over the past 25 years, the sublethal effects of contaminant exposure have been assessed by measuring a particular response within aquatic animals (Vernberg and Vernberg, 1974; Holwerda and Opperhuizen, 1991). Typically, one or two physiological variables (e.g. heart rate, oxygen consumption, ventilation, urine output) are measured in test organisms exposed acutely to different concentrations of selected contaminants. While such an approach provides an insight into the responsiveness of the physiological system under investigation, it does not provide a holistic assessment of the impact of chemical toxicity on the whole organism. It is, after all, the integrated repertoire of compensatory responses which determines the survival potential of individuals, and impacts on populations and communities. The dearth of studies which simultaneously monitor changes in metabolic activity, cellular pathology, physiology and behaviour reflects partly the lack of suitable instrumentation, but also partly that workers carrying out studies at one level of biological organisation (e.g. the physiological level) are often not familiar with concurrent responses at other levels of organisation (e.g. molecular or behavioural levels). In recent years, however, some multi-disciplinary studies have been conducted, for example into the effects of hydrocarbons on various systems in *Carcinus maenas* (L.) (Bayne and Thurberg, 1988; Capuzzo and Leavitt, 1988; Lee, 1988).

1.1.2 Test organism (*Carcinus maenas*) and contaminant (copper)

The shore crab *C. maenas* was selected as a "model organism" since the single-system approach has been applied frequently to this common littoral species and has provided a wealth of background information. *Carcinus maenas,* an extremely eurythermal and euryhaline species (Taylor and Taylor, 1992), is distributed widely along the coastal areas of north-west Europe (Hayward and Ryland, 1990). Consequently, shore crabs may be exposed to a broad range of anthropogenic contaminants, yet remain common and abundant. This suggests that compensatory mechanisms which allow the crab to survive natural environmental fluctuations may also confer some degree of tolerance to contaminant exposure (Depledge and Bjerregaard, 1989a). In this chapter, some of the principal responses to waterborne copper at several levels of organisation in *C. maenas* are summarised to provide an integrated view of an organism's response (Fig. 1.1) and to provide a background for the work presented in the following chapters. Copper is selected as the contaminant for consideration as it is an essential trace element in crustaceans, playing a key role in growth and in the metabolic functioning of haemocyanin, the oxygen-binding haemolymph pigment (White and Rainbow, 1982; Spaargaren, 1983; Rainbow,

1985). Excess copper is, however, highly toxic to crustaceans and, in former mining areas such as Restronguet Creek, Cornwall (UK) (Bryan and Gibbs, 1983), it poses a potential threat to benthic decapods.

1.2 Behavioural Responses

When an organism comes into contact with a contaminant it either remains unaware of the change in environmental quality, or chemosensory perception initiates a suite of behavioural responses (Fig. 1.1). With regard to *C. maenas,* little is known concerning the thresholds of detection for copper, although instantaneous changes in heart rate (increase and subsequent decrease) (which indirectly signify stimulation of chemoreceptors) occur at 1 to 3 mg Cu L^{-1} (Depledge, 1984a; Aagaard *et al.*, in press). Once detected by the olfactory receptors on the antennules (Hara *et al.*, 1983), avoidance responses are usually initiated. This has been documented for stream invertebrates in response to copper algicides (Luederitz *et al.*, 1989). While avoidance behaviour *per se* has not been reported for *C. maenas*, locomotor activity increases markedly following exposure of shore crabs to 1 mg Cu L^{-1} (Aagaard and Depledge, 1993). Behavioural changes, resulting from direct stimulation of receptor sites, may lead to a disruption in mating behaviour and feeding response (Fig. 1.1) (e.g. due to competition between the contaminant and naturally occurring chemical stimuli) (Hara *et al.*, 1983). Such effects have been reported for another intertidal crab, *Pachygrapsus crassipes* Randall, which experiences inhibition of the feeding response (to taurine) and fails to display the male mating stance response (to female sex pheromone) following exposure to crude oil extracts (Takahashi and Kittredge, 1973). A review of the behavioural effects of contaminants on aquatic organisms has been compiled by Hara *et al.* (1983).

1.3 Cellular Responses

Prolonged exposure to waterborne copper results in copper uptake (Wright, 1995) and the binding of copper ions to molecular sites within the organism (Rainbow, 1995). The gills present a relatively large permeable surface for the exchange of waterborne chemicals and are the first system to be impacted. As the shore crab *C. maenas* inhabits aquatic environments with a wide range of salinity fluctuations, the gills play an important role in enabling this extremely euryhaline species to colonise dynamic environments, as well as allowing the uptake of oxygen and the excretion of CO_2 and nitrogenous wastes. Their role as the main site of exposure to any waterborne contaminants, therefore, has important repercussions on the physiology of the animal.

1.3.1 The phyllobranchiate gill of *Carcinus maenas*

Although there are numerous publications describing the general gill ultrastructure of *C. maenas* (e.g. Ali, 1966; Goodman and Cavey, 1990; Lawson *et al.*, 1994), a brief description is given here to facilitate the understanding of the following chapters, which deal with various aspects of gill ultrastructure and function.

Carcinus maenas has 9 pairs of phyllobranchiate gills, obscured by the lateral extensions of the carapace, the branchiostegites (Figs 1.2a and b). The gills are divided into functional types. A mainly respiratory function is attributed to the six anterior (smaller) gills, whereas the posterior gills (numbers 7-9) of each gill chamber are responsible mainly for iono- and osmoregulation (Compère *et al.*, 1989). Each gill is made up of a series of lamellar plates, which become smaller with increasing distance from the site of attachment, and are covered with a thin cuticle (0.7-1.5 μm) (Fig. 1.3a). The two lateral lobes of each lamellar plate are connected by the central raphe, at the epibranchial end of which is situated the afferent haemolymph vessel (Fig. 1.3b). Lamellar arteries radiate from the afferent haemolymph vessel into the tissue. The haemolymph travels to the hypobranchially situated efferent vessel via the marginal canal and a series of efferent channels (Taylor and Taylor, 1986).

Generally, a layer of algal/bacterial cells is found on the gill cuticle surface (Figs 1.4a to c). On the interior side of the two cuticle plates, which generally are thinner in anterior (*c.* 800 nm) than posterior gills (*c.* 1100 nm), epithelial cell layers are found, between which the flow of haemolymph occurs. Cell types present and their form and function are summarised in Appendix 2. Anterior and posterior gill epithelial cell layers differ (leading to the distinction between respiratory and osmoregulatory gills, as discussed above), the former consisting of a predominantly thin layer of respiratory chief cells (Figs 1.4d and e). These cells contain elaborately folded apical plasmalemma. In gills from crabs collected from uncontaminated sites, there is a degree of vacuolation observed in all epithelial layers (Fig. 1.4f). Due to the relatively thin epithelial layer, the haemocoel remains fairly unobstructed, and contains few haemocytes and intralamellar septa, as well as pillar cells which generally connect the two sides of the lamella (Figs 1.4d, g and h). In anterior and posterior gill lamellae, pillar cells provide structural support, strengthened by the microfilaments transgressing the cells (Fig. 1.4i), as well as playing a role in the direction of haemolymph flow (Taylor and Taylor, 1986). Haemocytes, present in respiratory and osmoregulatory gills, contain granules of various sizes (Figs 1.5a to c). Generally, the granules appear electron-dense, although they may have a honeycombed structure. Some

granules represent lysosomes, involved in the detoxification of metals (Viarengo, 1985), however, the functional distinction between granule types cannot be made by TEM. A number of different functions are attributed to haemocytes, including carbohydrate transport and metabolism (Johnston *et al.*, 1973), amino acid storage (Evans, 1972), lipoprotein transport (Sewell, 1955) and cellular defence (Smith and Ratcliffe, 1980). Although a direct involvement in metal detoxification has been shown in other decapods (Balaji *et al.*, 1989), this has not been confirmed for the haemocytes of *C. maenas* (Rtal and Truchot, 1996). Frequently, the lamellar septum is combined with the presence of haemolymph capillaries (Fig. 1.5d). Posterior gill epithelia consist predominantly of striated (Fig. 1.5e) and pillar cells (Fig. 1.4h). The former, which are thicker than chief cells and, therefore, reach further into the haemocoel, contain elaborate basal plasmalemma in combination with elongate mitochondria (a reflection of their role in ionic exchanges). Nephrocytes, easily identified by the pedicels around the cell margins, are more common in the haemocoel of posterior than anterior gill lamellae, and are frequently attached to the lamellar septum (Figs 1.5f and g). Nephrocytes are involved in endocytotic processes (Strangeways-Dixon and Smith, 1970; Taylor and Taylor, 1992).

Gaseous exchanges across the gills are achieved mainly by diffusion, although the membrane-bound enzyme carbonic anhydrase facilitates CO_2 excretion by catalysing the conversion of the HCO_3 pool to CO_2 (Taylor and Taylor, 1992). The osmoregulatory (posterior) gill epithelia differ in structure from the respiratory (anterior) gills, containing many basal membrane folds and associated elongate mitochondria (Fig. 1.5e). In addition, osmoregulatory gills have higher levels of Na,K-ATPase, the principal mediator of ionic exchanges across cellular membranes (Siebers *et al.*, 1982), than respiratory (anterior) gills.

1.3.2 The effect of exposure to copper

On entering the gill, copper binds to surface proteins (Webb, 1979; Evtushenko *et al.*, 1986). Uptake of trace metals into the crustacean gill cells and haemolymph occurs either passively down concentration gradients, with the aid of carrier proteins, and/or through the action of active transport pumps (Simkiss and Taylor, 1989). Uptake may result in ultrastructural gill cell damage as reported by Nonnotte *et al.* (1993) following exposure of *C. maenas* to 500 μg Cu L^{-1} in full-strength sea water. At this exposure concentration, severe epithelial cell damage was observed after five to six days, manifest as thickening of the gill epithelium, vacuolisation, cellular hyperplasia and necrosis. Similar structural alterations were observed by Lawson *et al.* (1995) at lower concentrations of copper (50 μg Cu L^{-1}). In addition, Lawson *et al.* (1995) demonstrated significant disruption of both the

apical and basal plasma membrane systems, and epithelial vacuolisation. Such ultrastructural gill damage has consequences for osmotic and ionic regulation, and for circulatory and respiratory physiology (discussed below).

Subsequent passage of copper ions bound to cellular ligands (Rainbow and Dallinger, 1993) through the gill epithelium delivers the copper to the haemolymph. Here metal ions bind rapidly to haemolymph proteins and haemocyte cell surfaces (Martin *et al.*, 1977; Depledge and Bjerregaard, 1989b). Exposure to various levels of copper (250, 500 μg Cu L^{-1}, 1 mg Cu L^{-1}) is also associated with decreasing haemocyanin concentrations in the haemolymph (Bjerregaard and Vislie, 1986). The proposed cause is an increase in haemolymph volume rather than a change in overall haemocyanin levels (Depledge and Bjerregaard, 1989b), possibly linked to a change in apparent water permeability of the animal (Rasmussen *et al.*, 1995). Copper ions, entering the intracellular compartment of gill cells, bind to intracellular structural proteins and chemical messengers resulting in the triggering of cellular detoxification mechanisms (Engel and Brouwer, 1989). Detoxification of reactive metal species is achieved by the synthesis of metallothioneins (MT), low molecular weight (ca. 6-10 kDa), metal-binding proteins (Engel and Brouwer, 1989). Synthesis of MT is induced by a variety of trace metal species (including Cd, Cu, Zn) once the "first line of defence" (the tripeptide glutathione) is no longer able to protect the cell from metal ion toxicity (Singhal *et al.*, 1987; Brouwer and Brouwer-Hoexum, 1991). Recently, metallothionein concentrations have been measured in the gills and midgut gland of *C. maenas* following chronic exposure to copper in Restronguet Creek (Pedersen and Lundebye, 1996), where levels reach 2.5 mg Cu g^{-1} in the sediment (Langston *et al.*, 1995). Values in gill tissue reflected the copper, zinc and cadmium concentrations in the sediment. In the midgut gland, however, tissue metal concentrations and MT concentrations were less closely correlated due to the confounding influence of the storage of metal rich granules in this tissue. Although both copper and zinc are normal cell constituents during metal homeostasis in crustaceans (Bryan, 1968), increased copper levels lead to a displacement of zinc (Engel and Brouwer, 1984) at the metal binding sites of MT. In midgut glands of blue crabs, *Callinectes sapidus* Rathbun, MT concentrations fluctuated with changes in the physiological state of the animal during the moult cycle (Engel and Brouwer, 1993).

Another acute response to copper (and indeed other stressors) involves the synthesis of stress proteins (Fig. 1.1). Their synthesis, initiated by the presence of damaged proteins (Ananthan *et al.*, 1986), is postulated to modulate cellular metabolism, protecting cells from

further damage (Sanders, 1993) by reducing the rate and amount of reactive metal species incorporated into biochemical pathways. The cellular stress response confers increased tolerance to toxicant exposure (Sanders, 1990). Following exposure of *C. maenas* to sublethal copper levels (100 μg Cu L^{-1}) for 14 days, Vedel and Depledge (1995) measured a significant increase in stress-70 in gill tissue. A review of the different families of stress proteins was published by Sanders (1993). Detoxification and cellular stress responses of gill tissue may not prevent some degree of metabolic disruption. Indeed, several gill enzymes exhibit altered activity levels in *C. maenas* following copper exposure. For example, Hansen *et al.* (1992b) observed rapid, and significant, reductions in the activity levels of key metabolic enzymes. The glycolytic enzyme hexokinase showed a 77% reduction and pyruvate kinase activity was undetectable following one day of exposure to 10 mg Cu L^{-1}. The activity of Na,K-ATPase, an enzyme essential to crustacean osmoregulatory ability (Siebers *et al.*, 1982), decreased by 50 to 60% following 7 days exposure to 10 mg Cu L^{-1} (Hansen *et al.*, 1992a).

Disturbances in gill function are accompanied by changes in other tissues and organs as copper is distributed throughout the body by the circulatory system. For example, copper disturbs lysosomal function in haemocytes (and presumably in other cells). Lysosomes are considered to be the principal site of metal compartmentation in the cells of marine invertebrates (Sternleib and Goldfischer, 1976). High concentrations of trace metals are accumulated within the lysosomes in non-toxic forms (e.g. bound to MT) and are subsequently removed, bound to lipofuscin granules, by exocytosis (Viarengo, 1985). The egestion of inorganic particles (which contain metals) with the faeces in *C. maenas* is a normal event in the feeding cycle of crabs (Hopkin and Nott, 1980). Surface reactivity of these particles is high (Taylor *et al.*, 1988), supporting their involvement in the detoxification processes (Simkiss and Taylor, 1994).

Excretion of trace metals occurs directly via the gill surfaces (Viarengo and Nott, 1993) and in the urine (Bryan, 1967). Although this has not been investigated in detail for copper, the gills are considered a major route for metal excretion in many aquatic animals (Wright, 1995).

1.4 Physiological Responses

Metabolic and cellular disturbances inevitably have physiological consequences (Fig. 1.1). Exposure of *C. maenas* to 1 mg Cu L^{-1} results in significant changes in haemolymph osmolality and cation/anion balance (Bjerregaard and Vislie, 1986; Johnson, 1988; Boitel

and Truchot, 1990). Bjerregaard and Vislie (1986) measured an 80 to 90% reduction in haemolymph concentrations of Na^+, K^+, Cl^- and total osmolality within 6 days of exposure to 1 mg Cu L^{-1}. Exposure to 500 µg Cu L^{-1} caused a similar reduction, but only at certain times of the year, indicating the involvement of the physiological state of the animal in determining its vulnerability to trace metal contamination. The functional disruption of the osmoregulatory enzyme Na,K-ATPase is implicated as the cause for the above changes in ionic and osmotic haemolymph parameters. A one-week exposure of *C. maenas* to 10 mg Cu L^{-1} reduced Na,K-ATPase activity by 50-60%, resulting in a 40% reduction in haemolymph sodium concentration (Hansen *et al.*, 1992a).

Depledge (1984a) reported a decrease in heart rate immediately following exposure of *C. maenas* to 10 mg Cu L^{-1}. Such changes may be the result of either direct toxic effects of copper, or represent a compensatory response which reduces the rate at which the toxicant is distributed throughout the body. Reduced gill perfusion occurs as a result of reduced cardiac activity, and gas exchange may be impaired further by gill tissue changes (Nonnotte *et al.*, 1993). In *C. maenas*, alterations in ventilation, circulation and the gas exchange surface (thickening) are manifest in a lowering of the $Pa(O_2)$ following exposure to sublethal levels of copper (Nonnotte *et al.*, 1993). Spicer and Weber (1992) found a similar response for another crab, *Cancer pagurus* (L.), but only in combination with hypoxia.

Gill ventilation changes at low exposure levels of copper have not been investigated in detail (but see review of Spicer and Weber, 1991). Spicer and Weber (1992) found that exposure of *C. pagurus* to 400 µg Cu L^{-1} did not cause significant changes in ventilation. Changes in ventilation/perfusion ratios were evident following exposure of *C. maenas* to 10 mg Cu L^{-1} (Depledge, 1984a). Impairment of gas transfer, e.g. due to cellular changes in the gill epithelium (Nonnotte *et al.*, 1993; Lawson *et al.*, 1995), as discussed above, may be the cause. In general, oxygen consumption of crustaceans decreases during exposure to heavy metals although there are exceptions (for a summary see Spicer and Weber, 1991). The effects are more pronounced when the metal contaminant is administered at low salinities, as oxygen consumption generally increases with decreasing salinity (Taylor, 1977).

1.5 Importance of a Holistic Approach

The previous review highlights the importance of an integrated approach in gaining an understanding of the effects of toxicants on the biology and ecology of crustaceans, and indeed, other animals. Relating the findings of studies of single systems (biochemical or

physiological) to the organism's overall performance is made virtually impossible by differences in experimental protocol (e.g. sample site; exposure concentration and duration; salinity), inter-individual differences (e.g. physiological condition), but most importantly, because the target tissue or system which was critical to the survival of the test species has usually not been identified. Experimental design must combine multiple measurements of variables which signal the functional status of each key physiological system, during a single experiment, to allow the assessment of the organism's integrated response to the toxic substance. Although single-system measurements offer an indication of the consequences of toxicant exposure (for example, a change in the activity of a particular enzyme), this may provide little insight into which organ, tissue or system is critically affected. Multi-system measurements, however, do address this issue. The advantages of the multi-system approach are highlighted by the following examples. Changes in glycolytic enzyme activity suggest that cellular energy deprivation may be the cause for the toxic effect of copper on crustaceans; however, simultaneous measurements of the cellular energy charge potential clearly shows that this is not the case (Hansen *et al.*, 1992b). Similarly, increased oxygen consumption following toxicant exposure may be attributable to the energetic demands of avoidance behaviour, but may also be an indirect effect of increased protein synthesis associated with detoxification (Calow and Sibly, 1990), i.e. due to the cost of stress resistance (Hoffmann and Parsons, 1991). Only a multi-system approach can resolve such issues.

A summary of the responses measured following exposure of *C. maenas* to copper in relation to exposure concentration and time (as available in the literature) is given in Figure 1.6. Clearly, actual responses will depend on exposure concentration and the duration of exposure. The literature available does not allow a precise sequence of effects at any one exposure concentration to be established. Nonetheless, the following general pattern of response will emerge.

As an immediate reaction to detection of the contaminant, the crab may attempt to leave the area (i.e. avoidance behaviour is initiated). If the contaminant interferes with chemoreception, other behavioural consequences may follow (cessation of feeding or failure to detect sex pheromones etc.). If avoidance does not occur and the crab remains in contaminated conditions, copper will enter the organism via the exchange surfaces of the gill. Early changes in cardiac and ventilatory activity may then occur. During acute exposure, effects of copper on the osmoregulatory ability of the crab may emerge, such as a reduction in the activity of the enzyme Na,K-ATPase and thus the functioning of the ion

exchange pumps. Within a few days of exposure, the cellular structure of the gills will begin to show signs of damage, adding to the impairment of osmoregulatory ability and inhibiting the exchange of gases between haemolymph and the surrounding water. Such structural changes may coincide with respiratory compensation, e.g. an increase in ventilation rates to increase the flow of water over the gills, however, oxygen tensions within the arterial haemolymph may well decline. Heart rate changes to compensate for the alteration in respiratory parameters. Simultaneously, detoxification mechanisms are activated, such as an increase in lysosomal activity and the formation of granules to excrete excess metal. Excretion of metal ions also occurs directly via the gill surfaces. At the molecular level, an increase in the synthesis (or conversion) of metallothioneins will be initiated, along with an increase in stress protein concentrations (such as stress-70). Since all these responses lie within the zone of compensatory reactions (Depledge, 1989a), repair of damage and restoration of function may be possible, even during continued exposure. Return to clean conditions obviously increases the chances of recovery. Continued exposure may push the animal beyond the limit of compensation and "curable" disability (Depledge, 1989a), leading progressively to death.

It is important to note that the repertoire of responses to stressors ultimately determines whether an organism can compete sufficiently well, both intraspecifically and interspecifically, to grow and reproduce. This is perhaps the most compelling argument for studying the integrated responses of organisms to diverse stressors. It has been suggested that a reduction in an organism's Darwinian "fitness" can be measured in terms of changes in its "scope for growth" (SFG) (Bayne *et al.*, 1979). SFG is defined as the balance of energy intake (absorption) and energy expenditure (i.e. respiration, excretion, reproduction). Although SFG measurements on *C. maenas* are currently being conducted (Sims and Depledge, unpublished), reductions in feeding (during exposure to copper) almost certainly result in a decrease in the absorption/expenditure ratio, to the detriment of growth and reproduction. Naylor *et al.* (1989) determined the effects of zinc on the energy budget of the freshwater amphipod *Gammarus pulex* (L.) and linked the consequent reduction in SFG with a reduction in food absorption. A SFG assessment represents only part of a suite of measurements needed to understand the impact of contaminants on the integrated response of an animal, and related consequences for populations and communities.

1.6 Research Objectives of the Thesis

The research presented in this thesis investigates the effects of sublethal exposure to waterborne copper, at three different levels of biological organisation, in the shore crab *Carcinus maenas*. The three levels chosen include gill tissue ultrastructure, metallothionein levels in gill (and midgut gland) tissue as a molecular biomarker of trace metal exposure, and, thirdly, scaphognathite activity as a variable of respiratory physiology. Relative sensitivities and the usefulness of the three factors as indicators of trace metal exposure are evaluated.

1.6.1 Gill ultrastructure

The main objective of the ultrastructural gill study was to establish how ultrastructural damage caused by exposure to copper was related to changes in metallothionein levels and changes in respiratory physiology, in particular scaphognathite activity. To allow this objective to be met, the threshold level of copper exposure which would result in marked cellular changes in the gill of *C. maenas* was investigated. Previous work had involved exposure of *C. maenas* to either relatively low (50 μg Cu L^{-1}, Lawson *et al.*, 1995) or high (500 μg Cu L^{-1}, Nonnotte *et al.*, 1993) concentrations of copper. Further objectives were to determine if anterior (respiratory) and posterior (osmoregulatory) gills are equally sensitive to copper exposure; to assess the sensitivity of *C. maenas* to low-level exposure (50 μg Cu L^{-1}) in a flow-through seawater system as opposed to a static exposure system; and to compare the effects of trace metal exposure on gill ultrastructure as observed in the laboratory with gill ultrastructure of crabs taken from field sites with different histories of trace metal contamination.

1.6.2 Scaphognathite activity

The main objective in the measurement of ventilatory activity in *C. maenas* was to establish if the cellular damage observed in the gills affected scaphognathite movements. Ultrastructural gill damage had been shown previously to affect respiratory haemolymph parameters (Nonnotte *et al.*, 1993). A second objective was to evaluate the use of the Computer Aided Physiological Monitor (CAPMON) (Depledge and Andersen, 1990), a non-invasive monitoring system, for the recording of scaphognathite movements. CAPMON had only been used previously for the detection of cardiac activity in invertebrates (e.g. Bamber and Depledge, in press).

1.6.3 Metallothionein

Metallothionein measurements were made to establish if effects of copper exposure on the gill ultrastructure and ventilatory physiology of *C. maenas* were met by the detoxification

action of this metal-binding protein, i.e. if responses to exposure occurred simultaneously at the molecular, cellular and physiological level.

1.7 An Outline of the Thesis

Chapter 2 investigates the effects of low-level copper exposure (50 μg Cu L^{-1}) on the gill ultrastructure of *C. maenas* when administered in a flow-through seawater system.

Chapter 3 establishes the threshold level of copper exposure, administered in a static seawater system, which creates ultrastructural change in the gills of *C. maenas* and assesses the relative sensitivity of anterior (respiratory) and posterior (osmoregulatory) gills.

Chapters 4, 5 and 6 relate ultrastructural gill changes to changes in scaphognathite activity and gill tissue metallothionein levels for *C. maenas* exposed to 500 μg Cu L^{-1} in a static system. In particular, Chapter 4 validates the use of CAPMON for the measurement of ventilatory activity in *C. maenas*, and assesses the effects of a 10 day exposure period on gill ultrastructure, scaphognathite activity and metallothionein levels. Chapter 5 investigates the effect of temperature increase (15°C to 21°C) on the scaphognathite activity of control and copper-exposed crabs. Results are discussed with reference to gill ultrastructure and metallothionein measurements. Chapter 6 assesses the effect of hypoxia (25% to 30% saturation) on the scaphognathite activity of control and copper-exposed crabs, with reference to cellular (ultrastructure) and molecular (metallothionein) changes in the gills.

Chapter 7 compares gill ultrastructure and metallothionein levels (gill and midgut gland) in two populations of *C. maenas* from two estuaries with different levels of trace metals.

Chapter 8 provides a general discussion of the main findings of the thesis, and offers suggestions for further research. The relative usefulness and sensitivity of the three levels of investigation (gill ultrastructure; ventilatory activity; metallothionein levels) are evaluated.

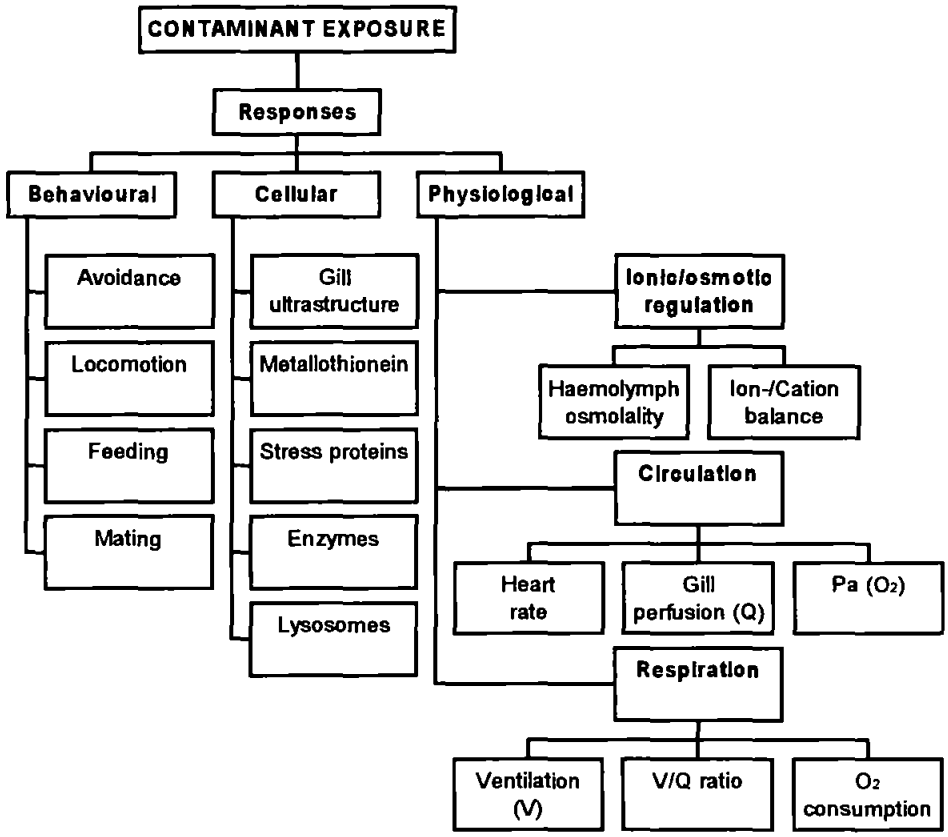


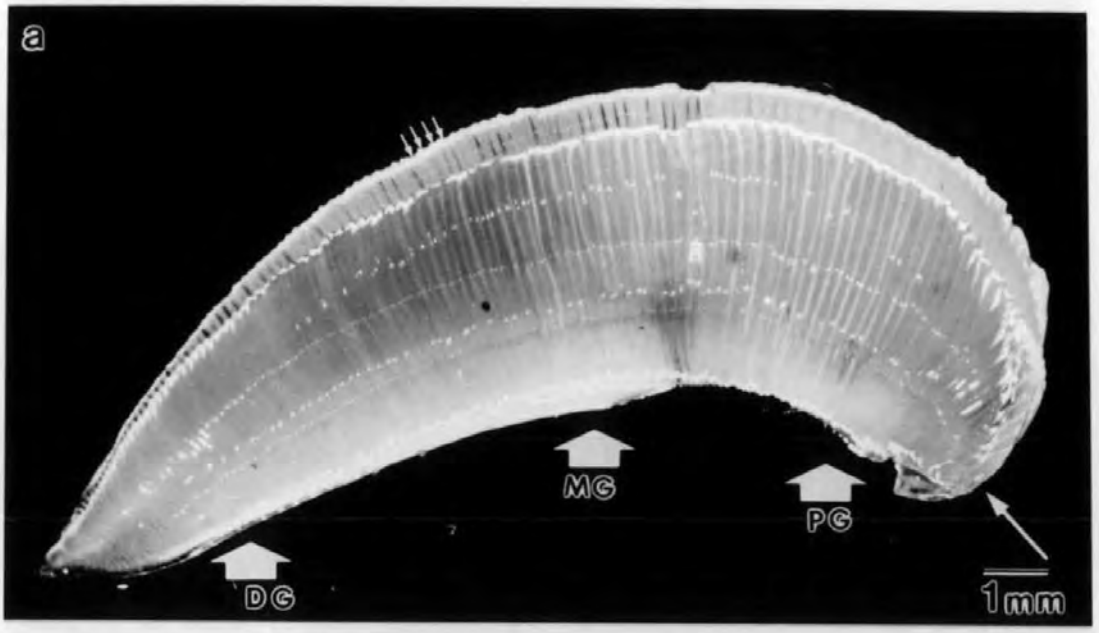
Figure 1.1: Compensatory responses of *Carcinus maenas* following exposure to copper.

Figure 1.2: a. Position of the right branchiostegite (BR) of *Carcinus maenas*. **b.** Partially dissected *Carcinus maenas* showing the position of the gills (G) within the right branchiostegite.


a
BR
1cm

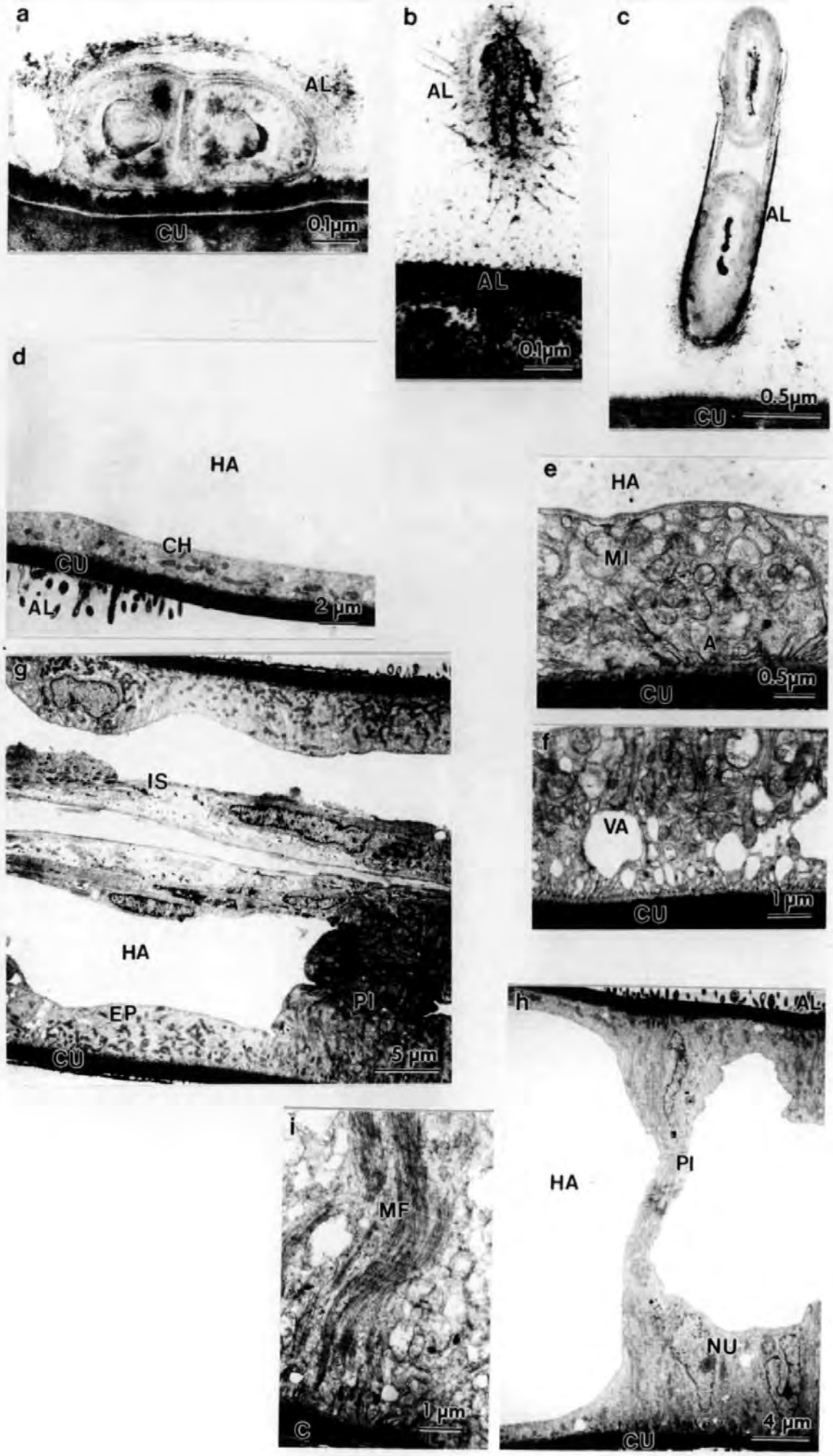

b
G
1cm

Figure 1.3: a. Lateral view of an entire posterior gill of *Carcinus maenas.* Small arrows indicate individual lamellar plates. Long, thin arrow indicates point of attachment within branchiostegite. Wide arrows indicate different gill zones: *DG*, distal gill region; *MG*, mid gill region; *PG*, proximal gill region. **b.** Transverse section through a posterior gill showing lamellar plates (wide arrows). Dotted line and arrow heads indicate ultramicrotome sectioning plane (i.e. TEM viewing plane). *AF*, afferent vessel; *CR*, central raphe; *E*, efferent vessel; *M*, marginal canal.


a
MG
PG
DG
1mm


b
M
AF
CR
E
0.5mm

Figure 1.4: Gill ultrastructure of *Carcinus maenas* from an unpolluted field site (Avon Estuary, Devon). **a-c.** Algal cells attached to the exterior of the gill cuticle. **d and e.** Chief cells of the thin respiratory epithelia of an anterior gill (No. 5). **f.** Vacuolated chief cell, illustrating the variability in cell condition found in control animals. **g.** Section through an anterior gill, showing both sides of the lamella and the intralamellar septum. **h.** Pillar cell spanning the haemocoel between the two sides of the lamella of an anterior gill. **i.** Microfilaments within a pillar cell. *A*, apical plasmalemma; *AL*, algal/bacterial layer; *CH*, chief cell; *CU*, cuticle; *HA*, haemocoel; *IS*, intralamellar septum; *MF*, microfilaments; *MI*, mitochondria; *NU*, nucleus; *PI*, pillar cell; *VA*, vacuole.


a
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c
AL
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AL
2 μm
e
HA
MI
A
0.5μm
CU
f
VA
CU
1 μm
g
IS
HA
EP
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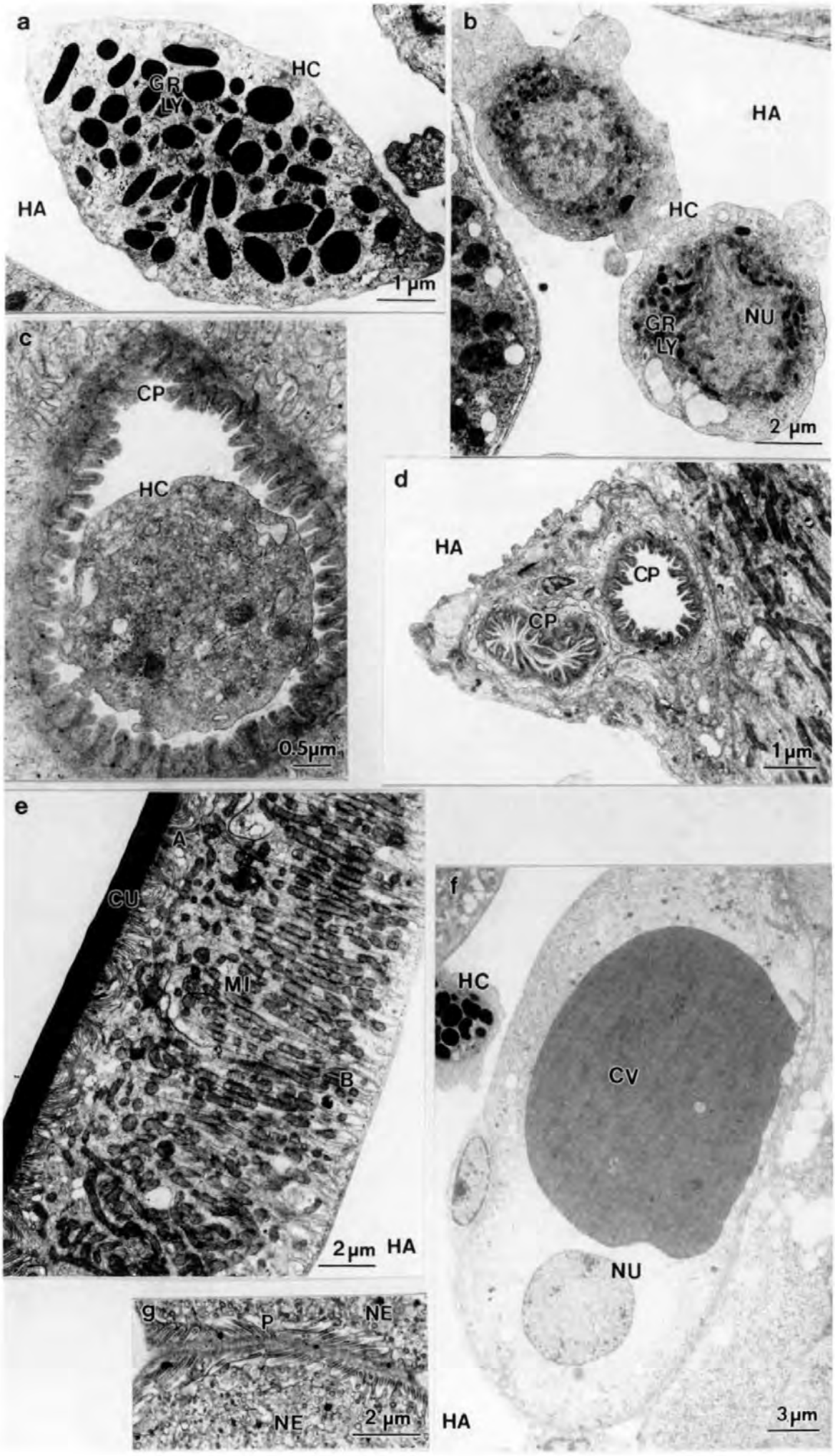
CU
5 μm
h
AL
PI
HA
NU
4 μm
CU
i
MF
1 μm
C

Figure 1.5: Gill ultrastructure of *Carcinus maenas* from an unpolluted field site (Avon Estuary, Devon). **a-c.** Haemocytes. **d.** Haemolymph capillaries in a posterior gill (No. 8). **e.** Striated cell in a posterior gill (No. 8). **f.** Nephrocyte in a posterior gill lamella. **g.** Pedicels of two bordering nephrocytes. *A*, apical plasmalemma; *B*, basal membrane; *CP*, haemolymph capillary; *CU*, cuticle; *CV*, central vacuole; *GR*, granule; *HA*, haemocoel; *HC*, haemocyte; *LY*, lysosome; *MI*, mitochondria; *NE*, nephrocyte; *NU*, nucleus; *P*, pedicels.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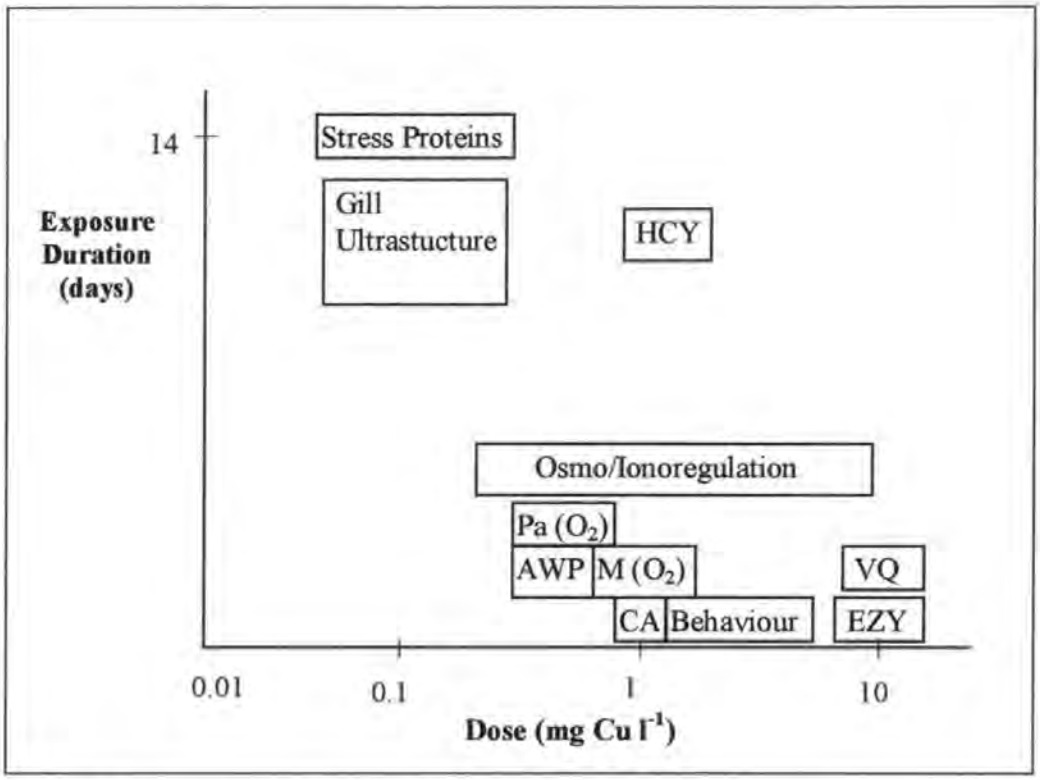


Figure 1.6: Diagrammatic representation of the literature available regarding relationships between dose/exposure duration and response types in *Carcinus maenas* following exposure to copper; apparent water permeability (AWP), cardiac activity (CA), enzyme activity (EZY), haemocyanin concentration (HCY), oxygen consumption (M (O_2)), arterial haemolymph partial oxygen pressure (Pa(O_2)), ventilation/perfusion ratios (V/Q).

CHAPTER 2

EFFECT OF COPPER ON THE ULTRASTRUCTURE OF RESPIRATORY AND OSMOREGULATORY GILLS OF *CARCINUS MAENAS*

Abstract

The common shore crab *Carcinus maenas* (L.) was exposed to sublethal concentrations of copper [0 (= control), 50, 100, 200, 300, 500 μg L^{-1}] for 14 days in a static laboratory system. At the lowest exposure concentration, the morphology of the anterior and posterior gills corresponded to that of control animals. Crabs exposed to 100 and 200 μg Cu L^{-1} showed anterior gill damage only. At the lower copper concentration, necrosis, and apical and mitochondrial vacuolation was present in areas proximal to the marginal canal. At 200 μg Cu L^{-1}, vacuolation was observed throughout the lamellae and epithelial hyperplasia seen in areas proximal to the marginal canal. At this copper concentration, a marked increase in the number of haemocytes, linked to infiltration of the epithelial layer by large-granule haemocytes, had occurred. At 300 μg Cu L^{-1}, the posterior gill epithelia appeared normal but contained lipofuscin granules, and the anterior gill epithelia were highly disorganised, with severe necrosis and hyperplasia throughout the lamellae. At 500 μg Cu L^{-1}, the posterior and anterior gills showed clear exposure effects, including the presence of lipofuscin granules of varying size (present within the posterior gill epithelia) and an increase in apical vacuolation, especially in thin epithelial zones. In these latter zones, the epithelial cells were partially necrotic, infiltrated by haemocytes and generally disorganised. Anterior gills were highly hyperplasic and contained large numbers of haemocytes (often vacuolated), which, at times, appeared to block the haemolymph channel. At this highest exposure concentration, epithelial cell structures were destroyed either by extreme necrosis or vacuolation, the latter resulting in detachment of the epithelial layers from the endocuticle.

The results indicate that the anterior gills were more sensitive to copper exposure than the posterior gills, and that within the gill lamellae, the area closest to the marginal canal showed greater damage than areas distant from the marginal canal. In addition, with decreasing exposure concentration, the variability of cellular ultrastructure (healthy or damaged) increased. These ultrastructural findings are discussed in relation to their functional implications.

2.1 Introduction

Trace metals, omnipresent in the marine environment, generally occur at very low levels unless there are anthropogenic inputs (e.g. from mining, or chemical and metal-processing) (Clark, 1992). For example, copper is present in oceanic waters at a concentration of approximately 0.1 μg L^{-1} (Brutland and Franks, 1983), although higher levels occur in the North Sea (0.2-2.6 μg Cu L^{-1}) and some UK estuaries (2-3 μg Cu L^{-1}) (Duinker and Nolting, 1982; Morris, 1984). Although copper is an essential trace element for aquatic crustaceans (e.g. in growth and for the functioning of the oxygen-binding haemolymph pigment haemocyanin) (Table 2.1), it is highly toxic when present in levels exceeding the beneficial range (Martin *et al.*, 1977; White and Rainbow, 1985; Harris, 1991). Species living in coastal and estuarine areas, like *C. maenas*, where anthropogenic inputs are most common, are often exposed to increased levels of copper. For example, populations of *C. maenas* in Restronguet Creek in Cornwall where mine wastes enter the estuarine environment, may have to tolerate copper levels as high as 176 μg L^{-1} (Bryan and Gibbs, 1983).

Table 2.1: Homeostatic copper concentrations in the tissue and exoskeleton of (fed) *Carcinus maenas* from unpolluted conditions (from Depledge, 1989b).

Tissue	*Gill* [$\mu g\ g^{-1}$ *DW*]	*Midgut gland* [$\mu g\ g^{-1}$ *DW*]	*Rest soft parts* [$\mu g\ g^{-1}$ *DW*]	*Exoskeleton* [$\mu g\ g^{-1}$ *DW*]	*Haemolymph* [$\mu g\ ml^{-1}$]
Copper	125.2±9.8	22.4±7.1	68.2±10.8	4.3±0.3	50

Gills, situated at the interface between the external medium and the internal blood or haemolyph, are the first site of contact with any water-borne contaminant and represent one of the main routes of trace metal uptake (Rainbow, 1995). Numerous studies describing the effects of trace metals on gill morphology of invertebrates have been conducted, generally following exposure to relatively high levels of trace metals in solution (Table 2.2). Recurring themes in reported gill ultrastructural damage are a reduction in the number of mitochondria present and mitochondrial distension (Bubel, 1976; Couch, 1977; Papathanassiou and King, 1983; Papathanassiou, 1985; Lawson *et al.*, 1995), damaged membrane systems (Bubel, 1976; Couch, 1977; Papathanassiou and King, 1983; Doughtie and Rao, 1984; Papathanassiou, 1985), a reduction in the number, and deterioration in structure, of cytosolic organelles like ribosomes and endoplasmic reticula (Bubel, 1976; Papathanassiou and King, 1983; Papathanassiou, 1985; Lawson *et al.*, 1995), epithelial

vacuolation (Couch, 1977; Ghate and Mulherkar, 1979; Papathanassiou and King, 1983; Papathanassiou, 1985; Nonnotte *et al.*, 1993; Lawson *et al.*, 1995), necrosis (Couch, 1977; Nimmo *et al.*, 1977; Ghate and Mulherkar, 1979; Nonnotte *et al.*, 1993) and hypertrophy (Nimmo *et al.*, 1977; Nonnotte *et al.*, 1993). Changes in haemocyte distributions (Bubel, 1976; Nimmo *et al.*, 1977; Ghate and Mulherkar, 1979; Nonnotte *et al.*, 1993) have also been reported frequently. These ultrastructural responses have been detected following exposure of crustaceans to a wide range of trace metals at various concentrations (Table 2.2).

Investigations into the effect of copper on the shore crab *C. maenas* have involved the exposure to either relatively high or low levels of metal. This study, therefore, set out to establish the threshold levels of response to water-borne copper in terms of gill morphological damage [e.g. the occurrence of cellular hyperplasia and necrosis (Nonnotte *et al.*, 1993)].

Table 2.2: Summary of gill morphological studies in crustaceans following exposure to trace metals.

Species	Trace metal	Dose	Reference
DECAPODA			
Penaeus duorarum; Palaemonetes vulgaris	cadmium	75-300 μg L^{-1}; 1 mg L^{-1}; 5 mg L^{-1}	Nimmo *et al.*, 1977
Penaeus duorarum	cadmium	763 μg L^{-1}	Couch, 1977
Macrobrachium kistnensis; Caridina sp.	copper sulphate	50 μg L^{-1}; 100 μg L^{-1}	Ghate and Mulherkar, 1979
Palaemon serratus	cadmium	5, 25, 50 mg L^{-1}	Papathanassiou and King, 1983
Palaemonetes pugio	chromium	500 μg L^{-1}; 1,2 & 4 mg L^{-1}	Doughtie and Rao, 1984
Crangon crangon	cadmium	5, 25 & 50 mg L^{-1}	Papathanassiou, 1985
Scylla serrata	mercury; cadmium; lead; arsenic; selenium	96h LC_{50} values	Krishnaja *et al.*, 1987
Crangon crangon	mercury	17 μg L^{-1}	Andersen and Baatrup, 1988
Carcinus maenas	copper	500 μg L^{-1}; 2 mg L^{-1}	Boitel, 1990; Nonnotte *et al.*, 1993;
Carcinus maenas	copper	50 μg L^{-1}	Lawson *et al.*, 1995
ISOPODA			
Jaera nordmanni	copper; mercury; cadmium	1 & 10 mg L^{-1} 100 μg L^{-1} & 1 mg L^{-1} 10 & 20 mg L^{-1}	Bubel, 1976

2.2 Materials and Methods

2.2.1 Collection and holding conditions

In January and March 1996, intermoult male *Carcinus maenas* of approximately equal size (carapace width 5-7 cm) and weight (50-60 g), with green carapaces (rather than red to unify intermoult stage), were collected from the Avon Estuary, Devon, using drop nets baited with various species of fish (Figs 2.1 and 2.2). The crabs were transported immediately to Plymouth in buckets of seawater (salinity of 35) and given 3 days to acclimate to aquarium conditions (15°C; 12 h light/12 h dark) in a holding tank containing recirculated and biologically filtered seawater (salinity of 35). During acclimation, the crabs were fed with gamma-irradiated mussel (*Mytilus edulis)* on introduction and after 2 days. Following acclimation, five individuals were placed into each of the six aerated 20 L experimental glass tanks containing seawater (salinity of 35) with copper concentrations ranging from 0 (=control), 50, 100, 200, 300 to 500 μg Cu L^{-1}. Copper solutions were prepared by adding the appropriate volume of a copper chloride stock solution [$Cu_2Cl.2H_2O$ in water] to the experimental tanks. To minimise copper depletion during the experimental exposures, all tanks had been acid-washed, rinsed and filled with the appropriate copper solutions 48 h prior to the introduction of test organisms. Test solutions were renewed immediately prior to introduction of the crabs, and on every following second day. Crabs were exposed for 14 days, during which they were not fed. Water samples were taken immediately before and after a water change to determine if any copper depletion had occurred within each 48 h period. Analysis of the water samples by atomic absorption spectrophotometry (Varian SpectrAA 600) established that losses of copper over 48 h did not exceed 15%.

Following 14 days of exposure, 4 animals from each tank were weighed, measured, and an anterior (No. 5) and a posterior gill (No. 8) removed from the right gill chamber.

2.2.2 Gill tissue processing (transmission electron microscopy)

Gills (Fig. 1.3a) (n=4 for each treatment), removed by cutting at the gill base, were placed immediately in vials of primary fixative (5% glutaraldehyde, made up with 0.2 M Na-cacodylate buffer at pH 7.2), the osmolality of which had been adjusted to match the crab haemolymph (*c.* 950 mOsm). Osmolality measurements of fixative and haemolymph, extracted at the arthrodial membrane at the base of a walking leg using a Drummond pipette, were made using a vapour pressure osmometer (Wescor 5500). Lamellar dissection was carried out in a Petri dish containing 0.2 M Na-cacodylate buffer (pH 7.2). Tissue samples (of approximately 1 mm diameter) were taken from the lamellae of the

mid-gill region (Fig. 1.3a). The gill pieces were left in the primary fixative for 1 h at 4°C, washed with Na-cacodylate buffer twice (10 min each) and post-fixed in 1% osmium tetroxide for 1 h at 4°C. The pieces were rewashed in buffer (for a maximum of 12 h) and gradually dehydrated in a graded series of alcohol (30, 50, 70, 90 and 100%) at 20 min each; the absolute alcohol step was repeated three times. Infiltration of resin (Spurr, 1969) was achieved using a graded series of absolute alcohol and propylene oxide (3:1, 1:1, 1:3, pure), and propylene oxide and resin (3:1, 1:1, 1:3, pure) in succession, each step requiring a minimum of 12 h (maximum 24 h). The vials containing gill tissue and pure resin were left in a rotating mixer at room temperature for 48 h (with a single change of resin). Tissue pieces were transferred to rubber flat-embedding moulds, orientated to make the microtome sectioning plane available (i.e. by positioning the required tissue edge close to the end of the individual moulds) (Fig. 1.3b), and polymerised at 70°C for 8 h. Following polymerisation, both thick sections (0.5 μm for light microscopy- stained with methylene blue) and ultrathin sections [80-120 nm for TEM- stained with uranyl acetate and lead citrate (Reynolds, 1963)] were cut using a glass knife on a Reichert ultramicrotome and collected on copper grids (200 mesh). Ultrathin sections were examined using a Jeol 1200 transmission electron microscope operated at 80-100 kV.

2.3 Results

2.3.1 Exposure to 50 μg Cu L^{-1}

The ultrastructure of the anterior and posterior gill epithelia, observed qualitatively under the transmission electron microscope (TEM), was not altered compared with that of control animals (Section 1.3.1; Figs 1.4 and 1.5). There appeared to be no change in the frequency or appearance of haemocytes and nephrocytes. The former contained granules of varying sizes (Figs 1.5a to c) in control and copper-exposed gills. Nephrocytes were more common in the posterior than the anterior gills of copper-exposed and control animals (*cf.* Section 1.3.1).

2.3.2 Exposure to 100 μg Cu L^{-1}

Posterior gill tissue remained unaffected following exposure to 100 μg Cu L^{-1}. Changes did, however, occur in the anterior gill epithelia, predominantly in the area proximal to the marginal canal. In this region, necrosis and mitochondrial vacuolation were common (Figs 2.3a and b). Also, mitochondria were distended and contained cristae at the periphery rather than evenly distributed throughout the mitochondrion (Fig. 2.3c). Necrosis lead to partial detachment of the epithelium from the endocuticle (Fig. 2.3a). Infiltration of the

epithelia by haemocytes occurred, however, there was no apparent increase in overall numbers compared with control gills. With increasing distance from the marginal canal (towards the central raphe), the variability of epithelial cell structure seemed to increase; healthy-looking cells (i.e. without distended mitochondria, necrotic areas or hyperplasic cells) dominating the epithelium (Fig. 2.3d). There was no apparent change in the appearance or frequency of nephrocytes.

2.3.3 Exposure to 200 μg Cu L^{-1}

At this exposure level, the posterior gill epithelia corresponded to that reported for control animals (Section 1.3.1). Changes in the anterior gill epithelia were especially pronounced in the area proximal to the marginal canal, where severe necrosis masked epithelial cell structures (Fig. 2.4a). Necrotic areas were overlaid by multinucleate hyperplasic tissue, formed by layers of degenerate haemocytes and electron-dense (possibly lysosomal) granules released from the damaged haemocytes (Fig. 2.4a). Non-necrotic epithelial cells were vacuolated and contained accumulations of glycogen (Fig. 2.4b). In contrast, respiratory epithelia distal to the marginal canal appeared unaltered. The appearance of the respiratory epithelia, however, varied greatly, and not all lamellae showed signs of necrosis and hyperplasia. Generally, increased numbers of especially large-granule haemocytes, which were often deformed due to the presence of vacuoles within the cytosol and contained accumulations of glycogen, were present in the anterior gill lamellae (Fig. 2.4c).

2.3.4 Exposure to 300 μg Cu L^{-1}

The posterior gill epithelia retained the general ultrastructure of control gills following exposure (Section 1.3.1), however, small (diameter 0.3-1 μm) electron-dense areas, resembling lipofuscin granules, were visible within the epithelial cells (Fig. 2.5a). Infiltration of some pillar cells by large-granule haemocytes was observed (Fig. 2.5b). Striated cells with a "healthy" appearance were often present adjacent to such affected cells. Nephrocytes, often attached to the lamellar septum, appeared to be more frequent than in control samples (Fig. 2.5c). Throughout the lamellae, anterior gill epithelia appeared completely disorganised following exposure, lacking mitochondria or apical membrane folds, although nuclei could still be identified within the empty cytosol (Fig. 2.6a). Unidentified, oval accumulations of uniformly dark staining fine material were present, sometimes contained within epithelial vacuoles (Fig. 2.6b). Hyperplasia occurred simultaneously with necrosis and loss of epithelial structures (Fig. 2.6c).

2.3.5 Exposure to 500 μg Cu L^{-1}

Posterior and anterior gill epithelia showed considerable damage following exposure to this copper concentration. Throughout the length of the lamellae, the posterior gill epithelia contained electron-dense lipofuscin granules (Fig. 2.7a). In the regions distal to the marginal canal, the granules were generally no larger than mitochondria and had highly heterogeneous contents. In the same epithelial region, apical membranes were interrupted by increased vacuolation (Fig. 2.7b), although apparently unaffected cells were also present. Vacuolation was more severe in the thin epithelial regions proximal to the marginal canal than in the thick epithelial regions distal to the marginal canal (Fig. 2.7c). This vacuolation was accompanied occasionally by necrotic areas close to the cuticle, infiltration of the epithelial layer by large-granule haemocytes and a general disorganisation of the epithelium similar to the hyperplasia found in anterior gill tissues (Fig. 2.7d). In some cases, the thickened gill epithelia extended to the lamellar septum, making contact with the increased numbers of nephrocytes and thus filling the haemolymph space (Fig. 2.7e). The epithelia of the anterior gills were affected throughout the length of the lamellae, with cells being either severely necrotic (Fig. 2.8a) or virtually absent due to extreme vacuolation (Fig. 2.8b). Remaining nuclei had pulled away from the surrounding cytosol and appeared shrunk (Fig. 2.8c). Unidentified, oval accumulations of uniformly dark matter were present both within the tissue and seemingly free in the haemolymph space (Figs 2.9a and b). Large-granule haemocytes were found adjoining the necrotic layer, either as part of the hyperplasic tissue or singularly (Fig. 2.9c). Overall, haemocytes appeared to be more frequent in the exposed tissue than in control samples, the cells often appearing deformed due to large empty lateral vacuoles as well as extensions of the cytosol (Fig. 2.9d).

2.4 Discussion

Exposure to 50 μg Cu L^{-1} in the static system failed to elicit any obvious changes in gill ultrastructure compared with control gills. Exposure to this copper concentration has been reported to cause damage to the posterior gills of *C. maenas* when applied in a flow-through system (Lawson *et al.*, 1995). Crustaceans accumulate metals at a faster rate when exposed in a flow-through rather than a static system, and there is some indication that animals exposed in flow-through conditions are more sensitive to the contaminant than animals in a static system (Vernberg *et al.*, 1977). A flow-through system offers the advantage of maintaining a constant exposure level in the experimental tanks, however, the results of water sample analyses in the present work, coupled to frequent water changes,

indicate that copper depletion in the static system was minimal. Fluctuations in copper exposure concentration are, therefore, an unlikely explanation for the lack of ultrastructural change. Furthermore, the small level of copper depletion measured in the static system does not explain why the structural changes in the posterior gill ultrastructure reported by Lawson *et al.* (1995) (i.e. including severe vacuolation) were not observed here until exposure to 10x the concentration of copper used by Lawson *et al.* (1995) (i.e. 500 μg Cu L^{-1}). Other possible reasons (e.g. source of animals) for the difference in response are discussed in detail in the following chapter (Section 3.4).

At exposure concentrations exceeding 50 μg Cu L^{-1}, gills showed signs of ultrastructural response, including epithelial vacuolation, necrosis, and hyperplasia. Mitochondria were generally distended and hypertrophic tissue was infiltrated by haemocytes which were present in increased numbers. These ultrastructural changes became more common and pronounced with increasing copper exposure dose. Respiratory epithelia, however, were more readily affected by copper exposure (at least up to concentrations of 500 μg Cu L^{-1}) than osmoregulatory epithelia. This was the case in terms of the entire lamella from the mid gill region of an anterior (respiratory) gill, as well as different epithelial zones within a single posterior (osmoregulatory) gill lamella. The anterior (respiratory) gills showed cellular damage following exposure to 100 μg Cu L^{-1}, whereas the posterior (osmoregulatory) gills were not affected structurally until exposure to 300 μg Cu L^{-1}. Indeed, the posterior gill epithelia never showed the same degree of damage (total cellular degeneration) as the anterior gills. Previous studies have shown that cadmium retention in (excised) anterior gills was significantly higher than in posterior gills (Clausen *et al.*, 1993). Clausen *et al.* (1993) suggested that differences in the surface to volume ratios between the smaller anterior and the larger posterior gills may be the reason for differences in uptake, i.e. the greater surface to volume ratio in the former favouring greater passive uptake across the epithelia. Such uptake would be aided by the thinner cuticle of the anterior gills. Variation in the apparent sensitivity of gills, however, may be related to functional type rather than size. For example, this study has shown that copper affects the respiratory epithelia more readily than the osmoregulatory epithelia. Epithelial hyperplasia, necrosis and vacuolation were more frequent, and more extensive, in respiratory than osmoregulatory epithelia. This was true for respiratory gills as a whole, but also for the area close to the marginal canal in the posterior gills, which is suggested to have a respiratory rather than osmoregulatory function (Taylor and Taylor, 1992). The present work, therefore, indicates that respiratory function would be affected at lower

copper concentrations than the ability to osmoregulate (if a direct link between structural and functional change is assumed). Boitel (1990) did not report any difference in the effect of copper on *C. maenas* gills relating to epithelial type, however, this may be related to the lamellae chosen for investigation. Within the posterior gills, the distal gill lamellae contain mainly respiratory epithelia (Lawson, 1994), and exclusive analysis of these lamellae may indicate equal sensitivity to copper exposure of anterior and posterior gills. Boitel (1990) did not specify the gill area used.

In the present study, the direct toxicity of copper was reflected in the presence of necrotic areas (indicating cell death) and disrupted membranes, as well as mitochondrial degeneration. Excess copper may cause such damage by binding to surface proteins, encouraged by the strong affinity of copper for the sulphydryl groups of proteins (Viarengo and Nott, 1993). Binding of metals to surface proteins has, for example, been shown following exposure to cadmium (Webb, 1979). Mitochondrial degeneration in gill epithelia following exposure to trace metals has been reported in fish (Deung *et al.*, 1978; Crespo and Sala, 1986) and in a wide range of crustaceans (Bubel, 1976; Doughtie and Rao, 1984; Papathanassiou, 1985; Lawson *et al.*, 1995), and these organelles are thought to be a prime target for heavy metal effects. Disruption of mitochondrial membranes reduces their ability to produce ATP by oxidative phosphorylation, resulting in an increase in membrane permeability which may account for the distended appearance of the mitochondria following trace metal exposure (Bubel, 1976).

The increase in intracellular vacuolation, especially in the apical region of the posterior gill lamellae, following exposure to 500 μg Cu L^{-1} may be a reflection of increased pinocytic activity (i.e. transport of ions such as metals from the cuticle to the haemolymph), rather than actual gill damage (Papathanassiou, 1985). The large vacuoles present in the anterior gill epithelia following exposure, however, appear to represent acute damage, possibly caused by osmotic imbalance of the cells. In these areas, vacuolation and necrosis lead to the virtual detachment of the epithelium from the cuticle, suggesting complete dysfunction of the branchial tissue. The origin of the accumulations of glycogen, near the vacuoles and throughout the hyperplasic tissue, is unclear, however, it is possible that glycogen is released following the disintegration of haemocyte cell membranes. This suggestion is supported by the presence of large granules, clearly resembling the electron-dense granules (and lysosomes) present within the cytosol of intact haemocytes, throughout the tissue. The large (up to 10 μm in length), ovoid electron-dense bodies present throughout the hyperplasic tissue have been reported previously (Boitel, 1990),

although their origin remains uncertain. They may represent further "degenerative zones", i.e. necrotic tissue caused by either the cytotoxicity of the exogenous copper or mechanical damage due to osmotic dysfunction, however, their very uniform appearance does not resemble any epithelial cell. Their ovoid shape indicates that the fine granular material fills, or at least used to fill, cellular vacuoles. The ovoid aggregations may, therefore, represent cellular material in advanced stages of degradation.

At the highest copper exposure concentration, thickening of the epithelial layer due to hyperplasia as well as vacuolation was observed in the exposed anterior gill and in the respiratory regions of the posterior gill. These changes lead to an increase in the distance between the exterior medium and the internal haemolymph, affecting the diffusion capacity of the branchial tissue (Nonnotte *et al.*, 1993). Severe damage of the respiratory epithelia will lead to impairment of gaseous exchanges. Disruption of basolateral membranes, to which carbonic anhydrase is bound, as a result of tissue damage further indicates that the conversion of haemolymph HCO_3^- to excretable CO_2 may be reduced, a process catalysed by carbonic anhydrase at the haemolymph/epithelium interface; this conversion is thought to aid the excretion of CO_2 from the haemolymph (Taylor and Taylor, 1992). Blockage of the haemolymph channel within the lamellae by epithelial thickening, as well as increased numbers of nephrocytes and haemocytes, leads to disturbances of haemolymph flow [i.e. transport of O_2 to tissues bound to haemocyanin, as well as removal of CO_2 (as HCO_3^-)]. As the only return of haemolymph to the heart stems from the gills, Boitel (1990) suggested that this leads to tissue hypoxia throughout the animal. Indeed, tissue hypoxia is thought to be the cause of mortality at lethal levels of exposure to copper (Nonnotte *et al.*, 1993).

Nonnotte *et al.* (1993) linked gill ultrastructural alterations in *C. maenas*, following 4 days of exposure to 500 μg Cu L^{-1}, to measurements of oxygen tensions, pH and lactate concentrations in the arterial haemolymph. With the occurrence of epithelial hyperplasia, thickening and vacuolation, arterial haemolymph partial oxygen pressure (PaO_2) declined significantly (from 14.8 to 3.2 kPa), accompanied by a non-lactic acidosis (from pH 7.89 to pH 7.73). After 18 days of continued exposure, recovery of these parameters occurred. Indeed, Nonnotte *et al.* (1993) observed some ultrastructural recovery following this period, and reported zones of "increased cellular activity" proximal to the marginal canal, and the formation of new epithelial structures on the cuticle. Although physiological recovery after continued exposure to trace metals has been reported for *Cancer pagurus* (L.) (Spicer and Weber, 1992), there were no signs of ultrastructural recovery in

C. maenas in the present study after 14 days of continued exposure. The ultrastructural damage observed here makes it highly unlikely that the branchial tissue would function normally. The recovery reported by Nonnotte *et al.* (1993) may be the result of a decline in the water copper concentration over the 18-day exposure period, as replenishment of the copper was not carried out. In the current study, constant water changes guaranteed a relatively constant exposure level.

Some of the changes in gill ultrastructure (summarised in Figs 2.10 and 2.11) suggests activation of detoxification mechanisms following exposure. The electron-dense heterogeneous inclusions seen in the posterior gill epithelia were identified as lipofuscin granules, although some less heterogeneous areas may represent autophagic vacuoles containing partially degraded mitochondria (Bozzola and Russell, 1992). Multivesicular lipofuscin bodies contain undigested material derived from lysosomal activity, such as lipid peroxidation endproducts, and are a normal constituent of ageing cells (Bozzola and Russell, 1992) (although never seen in control intermoult gill epithelia). In marine invertebrates, lipofuscin granules are involved in metal homeostasis and detoxification (Viarengo, 1985; Viarengo and Nott, 1993), the exocytotically excreted granules containing increased levels of metals (George, 1983). For example, exposure of mussels (*Mytilus galloprovincialis*) to 40 μg Cu L^{-1} for 6 days, resulted in accumulation of lysosomal lipofuscin in the digestive gland, resulting from decreased glutathione (GSH) antioxidant activity (Viarengo *et al.*, 1990). Increase in lysosomal activity in the present study is implicated by the large number of large-granule haemocytes present in copper-exposed gills of *C. maenas*. Some of the electron-dense granules of these haemocytes may represent lysosomes (i.e. hydrolytic enzymes within bounding membranes), even if the distinction between haemocyte granule types cannot be made by transmission electron microscopy. Although lysosomes have not been observed as a normal constituent of gill epithelial cells, the infiltration of the epithelium by large-granule haemocytes, and the presence of probably haemocyte-derived "free" granules/lysosomes in the hyperplasic tissue, indicates their involvement in epithelial detoxification by providing sites of cellular metal compartmentation (Viarengo, 1985). Exposure to trace metal renders lysosomal membranes leaky, through the binding of copper to membrane proteins (see above), resulting in the escape of hydrolytic enzymes into the cytosol (Viarengo, 1985). These enzymes can lead to the destruction of the surrounding cells, which may account for the highly degenerate epithelia, as well as deformed haemocytes present within the haemocoel and degenerate haemocytes within the hyperplasic tissue. An increase in the number of

haemocytes within crustacean gill tissue following exposure to metals has been reported (e.g. Bubel, 1976; Lawson *et al.*, 1995). The increase in the number of large-granule haemocytes may reflect the relationship between lysosomal metal sequestration and binding of metals to metallothionein within the cytosol. Viarengo *et al.* (1981) reported an initial binding of copper to thioneins within the cytosol following exposure, and a consequent decrease coinciding with an increase in the lysosomal fraction, from where elimination by exocytosis is possible. In *C. maenas*, haemocytes are not directly responsible for the binding of exogenous metal within the haemolymph (Rtal *et al.*, 1996) [although this was demonstrated in the crab *Scylla serrata* (Balaji *et al.*, 1989)]. The influx of haemocytes is, on the one hand, interpreted as a sign of cellular degeneration (Bubel, 1976), and, on the other hand, as an increase in demand for energy reserves such as through the metabolism of carbohydrates (Johnston *et al.*, 1973). Alternatively, haemocytes may be simply "caught" within the gill tissue due to the narrowing of the haemolymph channel.

The increase in the number of nephrocytes in the posterior gill tissue suggests an increase in endocytotic processes (Strangeways-Dixon and Smith, 1970). This may be linked directly to the increase in metal levels in the haemolymph and/or the simultaneous appearance of necrotic inclusions within the epithelial cell layer which will be concomitant with an increase in cell turnover and thus cell debris. The cytosolic content of the nephrocytes, however, seems unchanged by exposure to metal.

In summary, exposure of *C. maenas* to copper resulted in ultrastructural gill damage at concentrations as low as 100 $\mu g\ L^{-1}$ in the anterior (respiratory) gill epithelia. At higher exposure levels (300 $\mu g\ L^{-1}$), changes were observed also in the posterior (osmoregulatory) gills, although localised to zones containing respiratory epithelia. The greater sensitivity to copper of the respiratory than osmoregulatory epithelia was related to functional differences in the epithelial cells; the ion-exchange mechanisms of the striated cells creating a degree of tolerance to metal ions in the osmoregulatory epithelia. The main features of the affected branchial tissue were severe hyperplasia (leading to a narrowing or blockage of the haemolymph channels), infiltration of the epithelia by large-granule haemocytes (which were present in increased numbers throughout the lamellae), cellular necrosis and vacuolation, as well as activation of detoxification mechanisms such as lipofuscin granule formation and nephrocyte influx (Figs 2.10 and 2.11).

The important question that needs to be asked when considering structural gill damage is: what is the significance of these changes to the "health" of the organism? This

requires the distinction between compensatory and deleterious responses (Spicer and Weber, 1991). The tissue damage observed appears to be sufficient to impair respiration in *C. maenas* and has been shown to do so (Nonnotte *et al.*, 1993). This does not, however, preclude any ecological significance of the changes resulting from sublethal trace metal exposure, i.e. effects on the "Darwinian fitness", especially in view of reports regarding recovery of decapod respiration during prolonged exposure to sublethal levels of copper (Spicer and Weber, 1991; Nonnotte *et al.*, 1993).

Without further physiological studies, it cannot be shown conclusively that the ultrastructural gill damage shown here is directly related to functional impairment. Changes in membrane structure (described as "damage") may be a means of reducing the uptake of metal into the tissues (Doughtie and Rao, 1984). However, the degree of "damage" observed in tissues, especially at the highest copper concentration (500 $\mu g\ L^{-1}$), make it unlikely that such an advantage will outweigh disadvantages of an impairment of normal functions.

Figure 2.1: a-c. Location of the sampling site (arrow) for *Carcinus maenas* in the Avon Estuary (Devon).

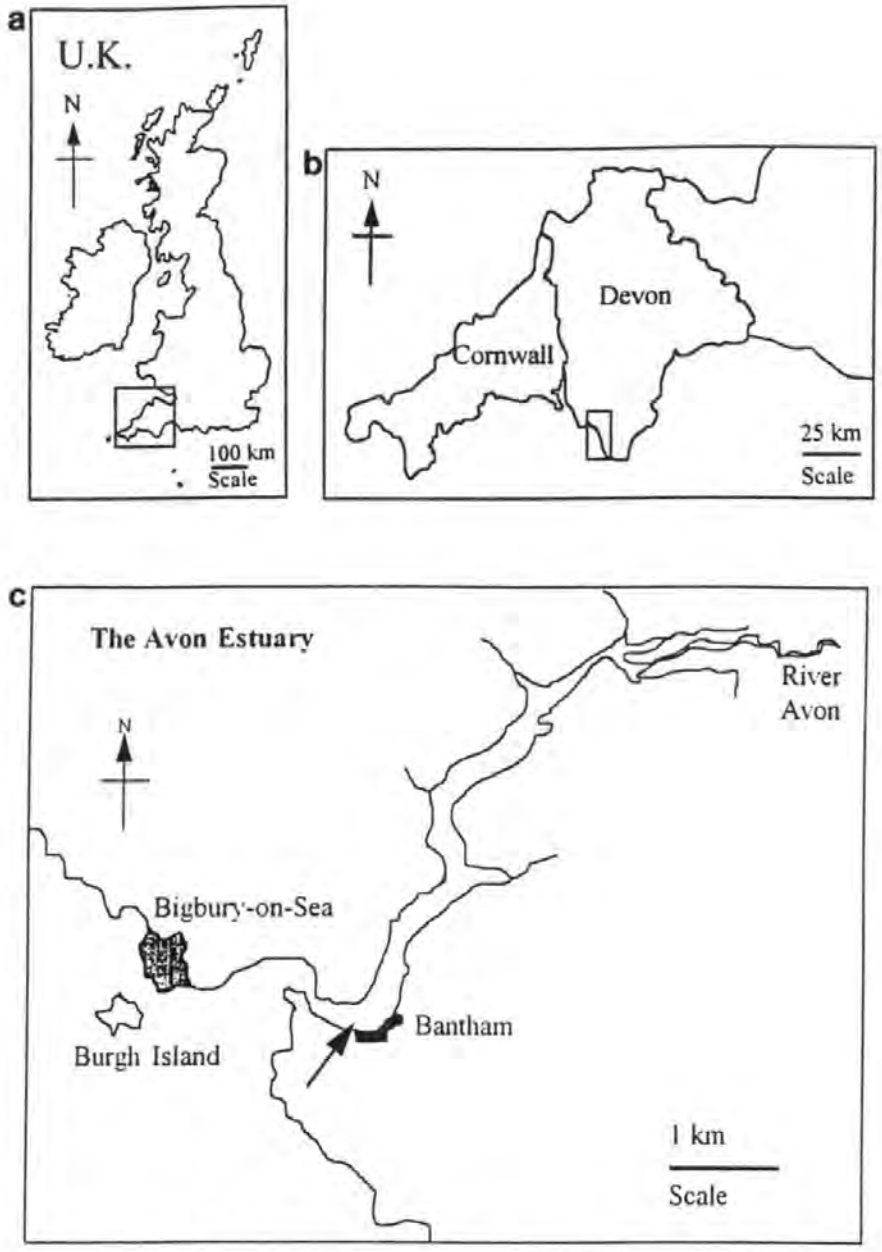

a
U.K.
N
100 km
Scale
b
N
Devon
Cornwall
25 km
Scale
c
The Avon Estuary
N
River
Avon
Bigbury-on-Sea
Burgh Island
Bantham
1 km
Scale

Figure 2.2: Drop net used for the sampling of *Carcinus maenas* (at rising and high tide), baited with fish (various species).

Figure 2.3: Anterior gill epithelia in *Carcinus maenas* following 14 days exposure to 100 μg Cu L^{-1}: **a.** Necrotic respiratory epithelium proximal to the marginal canal, arrow heads indicating electron-dense necrotic tissue, partly detached from the cuticle. **b.** Mitochondrial vacuolation (indicated by arrow heads) in both epithelia of a lamella proximal to the marginal canal. **c.** Distended mitochondria showing structural disorganisation, with cristae moved to the periphery of the organelles. **d.** Respiratory chief cell proximal to the central raphe with intact mitochondria. *AL*, algal/bacterial layer; *CE*, cristae; *CU*, cuticle; *HA*, haemocoel; *HC*, haemocyte; *IS*, intralamellar septum; *MI*, mitochondria; *NU*, nucleus.

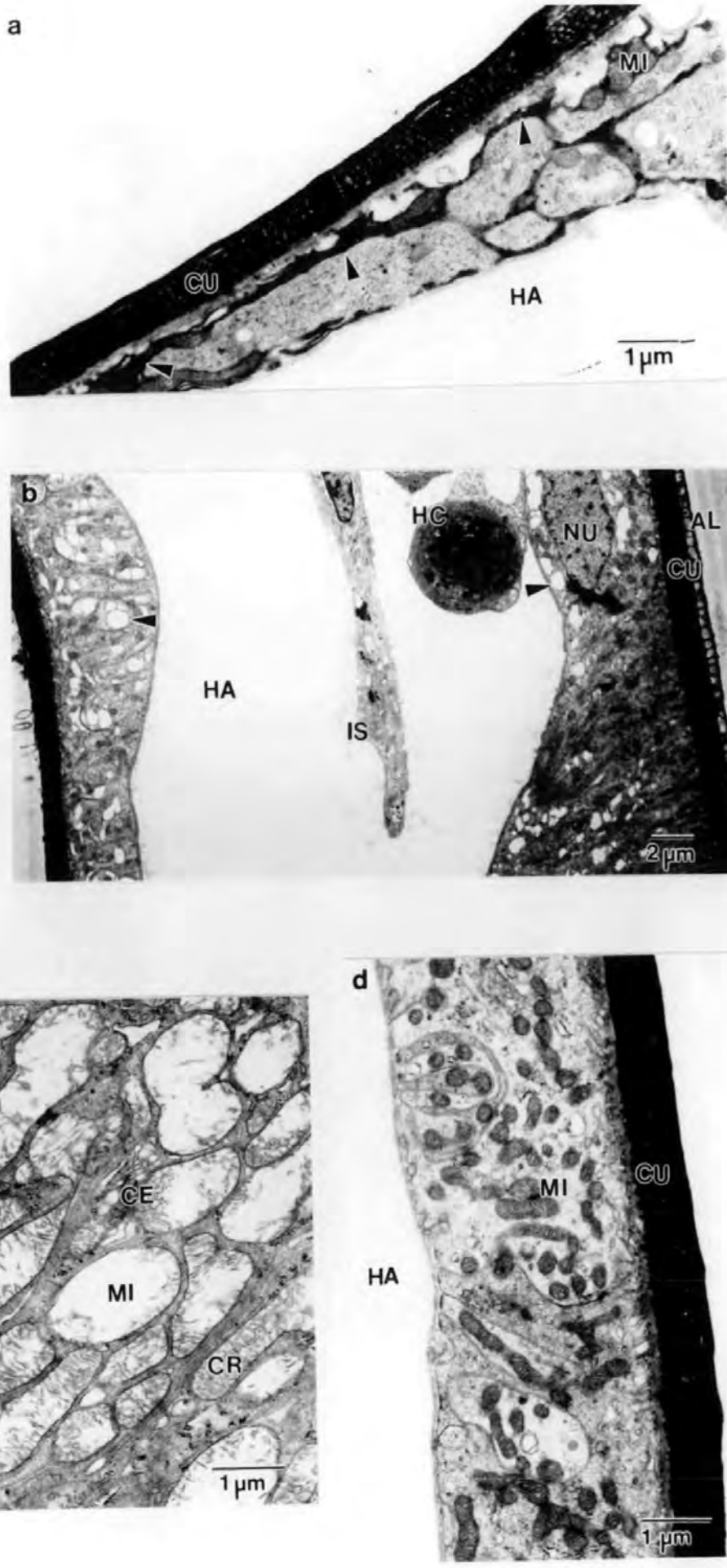
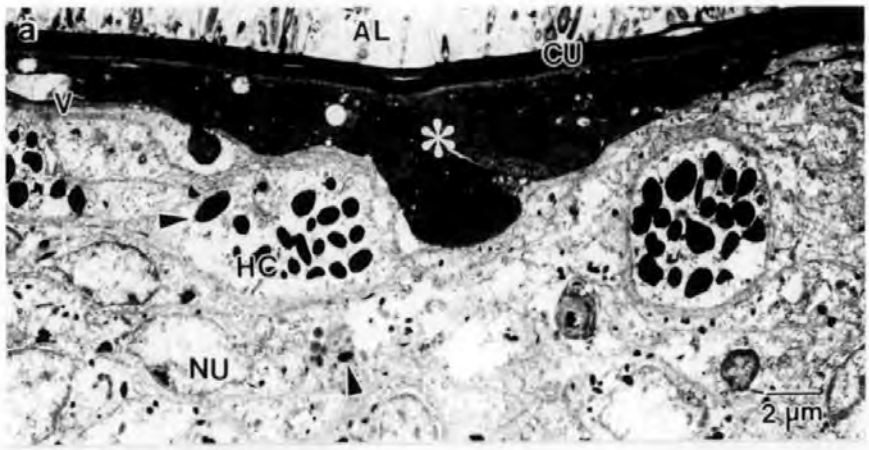
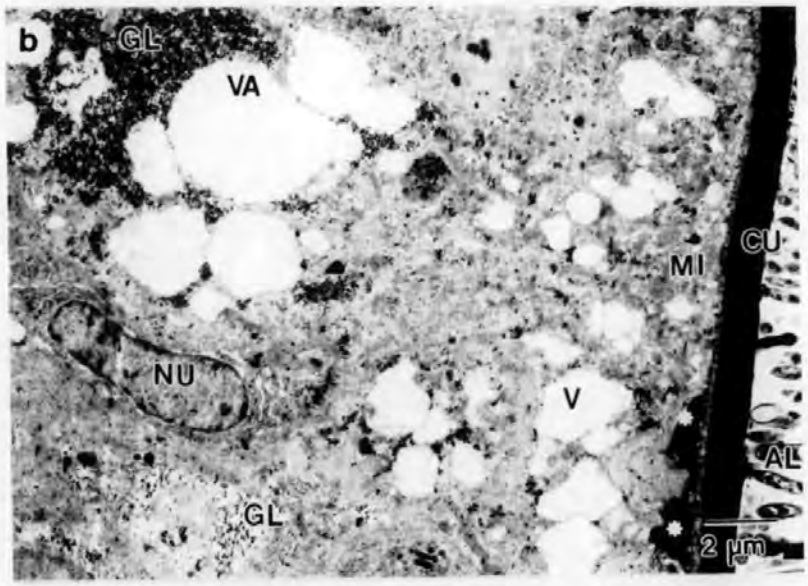

a
MI
CU
HA
1 µm
b
HC
NU
AL
CU
HA
IS
2 µm
c
CE
MI
CR
1 µm
d
CU
MI
HA
1 µm

Figure 2.4: Anterior gill epithelia in *Carcinus maenas* following 14 days exposure to 200 μg Cu L^{-1}: **a.** Severely necrotic epithelium proximal to the marginal canal, overlaid by multilayered hyperplasic tissue, containing degenerate haemocytes and (free) lysosomes/granules (indicated by arrow heads). **b.** Vacuolated epithelium with some necrosis and accumulations of glycogen. **c.** Degenerate haemocytes in the haemocoel, with cytosolic vacuole (arrow heads) and glycogen accumulations (small arrows). ✱/■, necrotic tissue; *AL*, algal/bacterial layer; *CU*, cuticle; *GL*, glycogen; *HA*, haemocoel; *HC*, haemocyte; *IS*, intralamellar septum; *LY*, lysosomes; *MI*, mitochondria; *NU*, nucleus; *VA*, vacuole.


a
AL
CU
V
HC
NU
2 µm


b
GL
VA
MI
CU
NU
V
AL
GL
2 µm

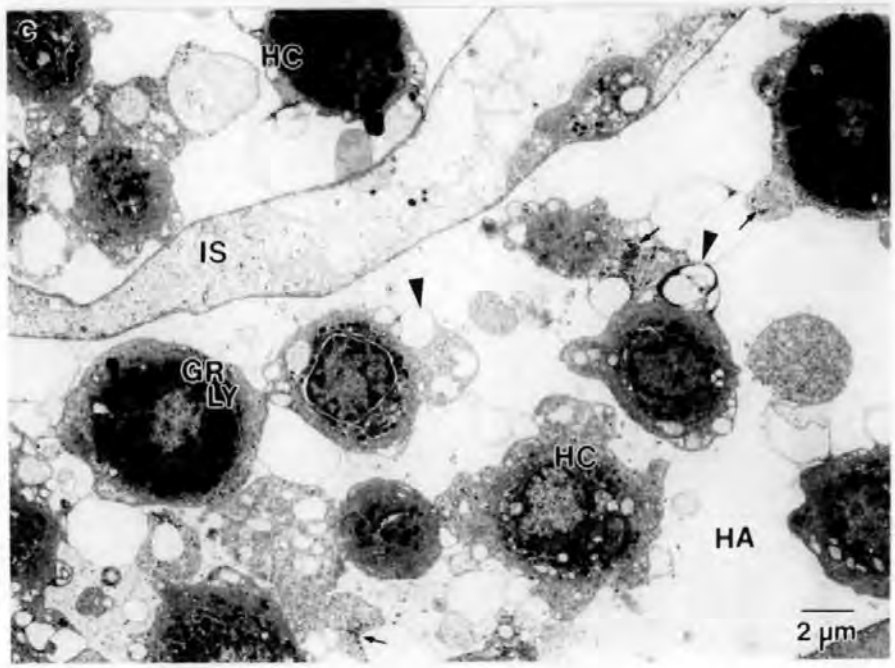
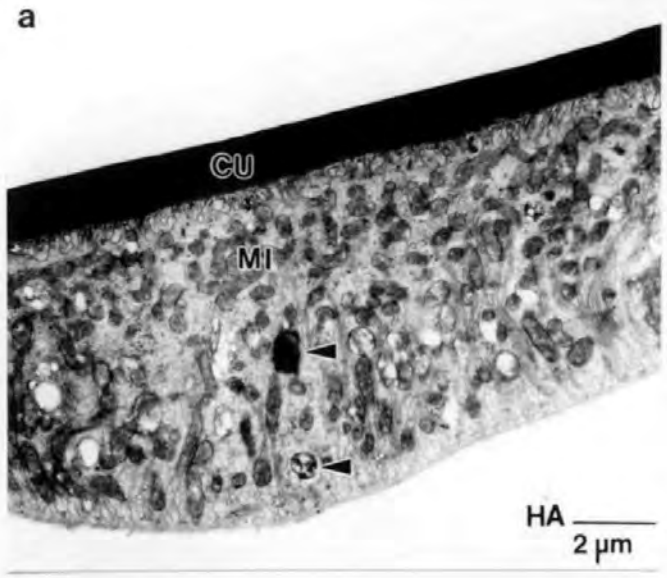
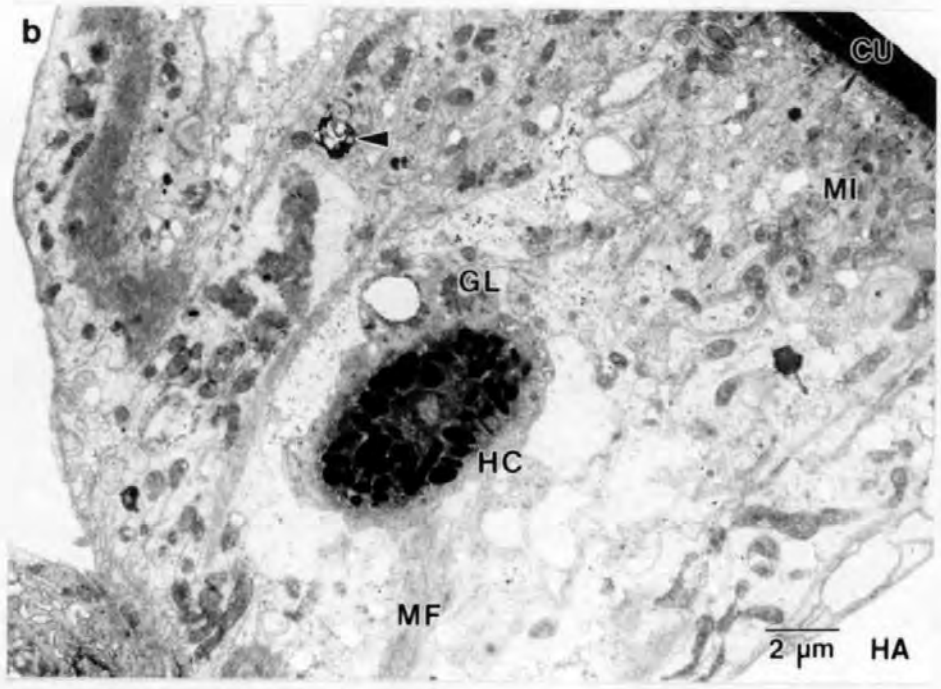

c
HC
IS
GR
LY
HC
HA
2 µm

Figure 2.5: Posterior gill epithelia in *Carcinus maenas* following 14 days exposure to 300 μg Cu L^{-1}: **a.** Striated cell proximal to the marginal canal containing electron-dense inclusions (arrow heads), resembling autophagic vacuoles or lipofuscin granules. **b.** Large-granule haemocyte with cytosolic glycogen within a pillar cell proximal to the marginal canal, containing electron-dense lipofuscin granules (arrow heads). **c.** Nephrocytes within the haemocoel, with characteristic pedicels (arrow head). *CP*, capillary; *CU*, cuticle; *CV*, central vacuole; *GL*, glycogen; *HA*, haemocoel; *HC*, haemocyte; *MF*, microfilaments; *MI*, mitochondria; *S*, satellite vacuole.


a
CU
MI
HA
2 µm


b
CU
MI
GL
HC
MF
2 µm
HA

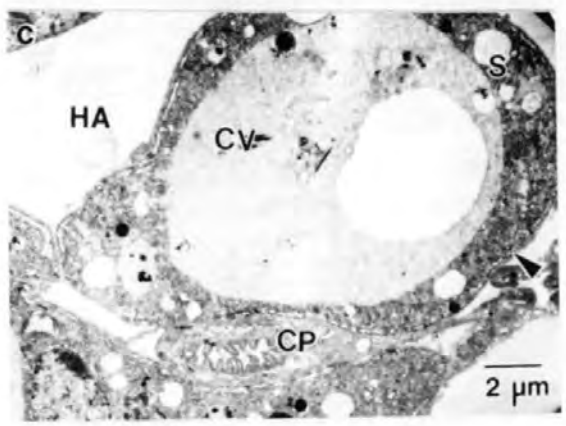
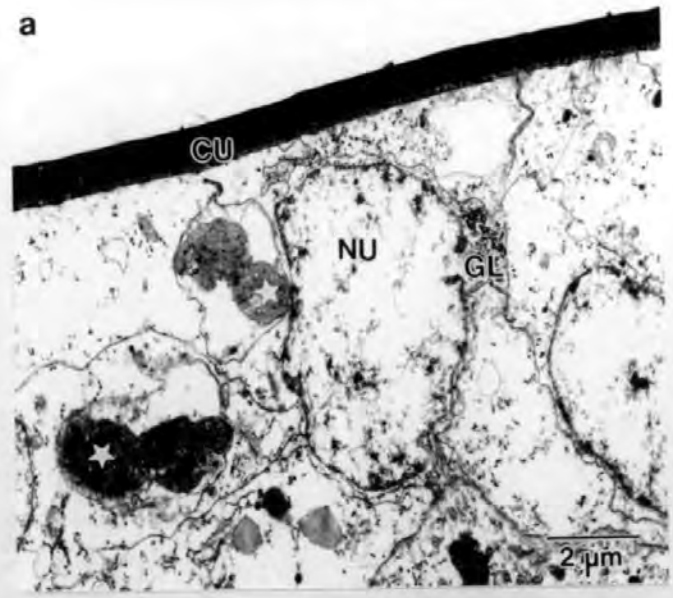
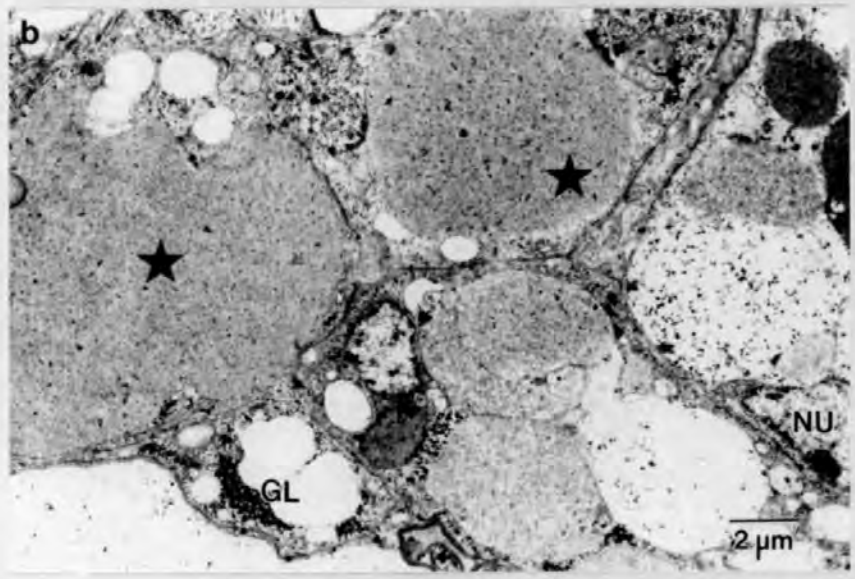

c
HA
CV
S
CP
2 µm

Figure 2.6: Anterior gill epithelia in *Carcinus maenas* following 14 days exposure to 300 μg Cu L^{-1}. **a.** Highly degenerate respiratory epithelium, devoid of mitochondria; mainly empty cytosol with glycogen accumulations and electron-dense inclusions (✪). **b.** Membrane-bound and non-membrane-bound electron-dense inclusions (★) within the hyperplasic tissue. **c.** Necrotic (✿) and hyperplasic tissue, containing degenerate haemocytes, free lysosomes/granules (arrow heads) and electron-dense oval inclusions (✦). *CU*, cuticle; *GL*, glycogen; *HC*, haemocyte; *NU*, nucleus.


a
CU
NU
GL
2 µm


b
NU
GL
2 µm

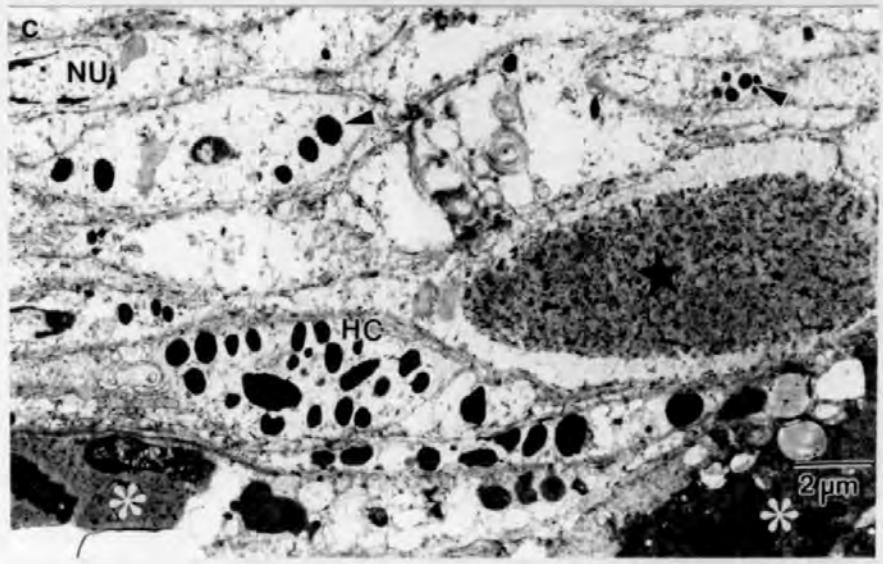

c
NU
HC
2 µm

Figure 2.7: Posterior gill epithelia in *Carcinus maenas* following 14 days exposure to 500 μg Cu L^{-1}. **a.** Multivesicular lipofuscin granule within the epithelium. **b.** Vacuolated apical plasmalemma. **c.** Disorganised epithelia proximal to the marginal canal, showing necrotic tissue (■), infiltration by large-granule haemocytes (degenerate), unidentified electron-dense inclusions (arrow heads) and free lysosomes/granules (small arrows). **d.** Vacuolation of thin epithelia proximal to the marginal canal. **e.** Nephrocytes [with characteristic pedicels (arrow head) and needle-like crystalline structures (arrow)] and epithelial cells meeting at the intralamellar septum. *A*, apical plasmalemma; *CP*, capillary; *CU*, cuticle; *CV*, central vacuole; *EP*, epithelium; *GL*, glycogen; *HA*, haemocoel; *HC*, haemocyte; *MI*, mitochondria; *NU*, nucleus; *VA*, vacuole.

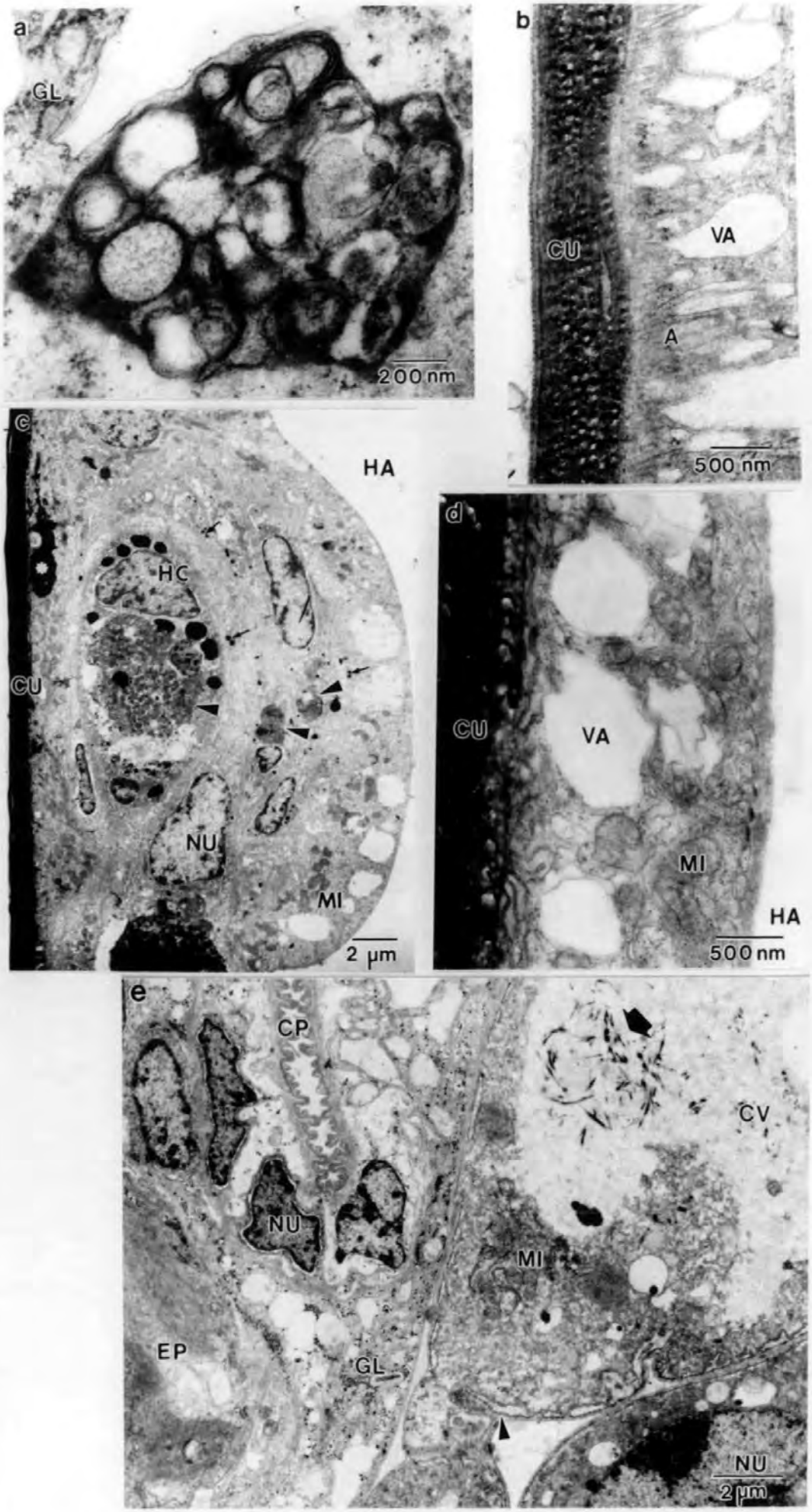

a
GL
200 nm
b
CU
VA
A
500 nm
c
HA
HC
CU
NU
MI
2 µm
d
CU
VA
MI
HA
500 nm
e
CP
CV
NU
MI
EP
GL
NU
2 µm

Figure 2.8: Anterior gill epithelia in *Carcinus maenas* following 14 days exposure to 500 μg Cu L^{-1}. **a.** Severe necrosis (▲) of respiratory epithelium. **b.** Vacuolation of the necrotic epithelium leading to virtual disappearance of the epithelial layer. **c.** Nucleus within the hyperplasic layers, showing shrinkage away from cytosol (arrow heads). *CU*, cuticle; *GL*, glycogen; *GR*, granules; *LY*, lysosomes; *NU*, nucleus; *VA*, vacuole.

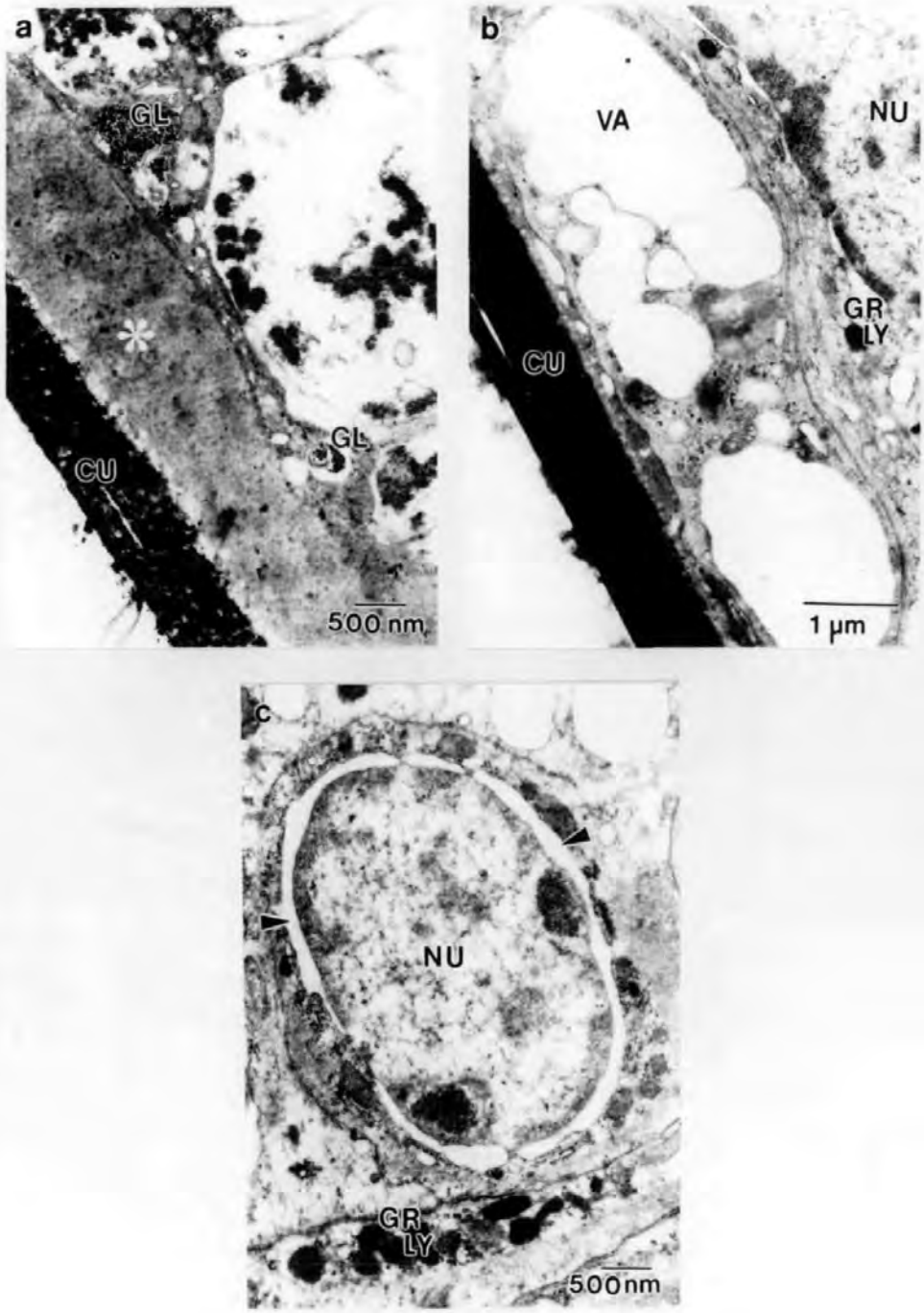

a
GL
GL
CU
500 nm
b
VA
NU
GR
LY
CU
1 µm
c
NU
GR
LY
500nm

Figure 2.9: Anterior gill epithelia in *Carcinus maenas* following 14 days exposure to 500 μg Cu L^{-1}. **a.** Oval-shaped inclusions within the hyperplasic tissue, representing break-down of necrotic epithelial cells (). **b.** Oval-shaped electron-dense inclusions () free in the haemocoel. **c.** Large-granule haemocyte adjoining epithelial necrotic layer (). **d.** Granular haemocytes within the haemocoel deformed by cytosolic vacuoles (arrow heads). *CU*, cuticle; *GR*, granules; *HA*, haemocoel; *HC*, haemocyte; *IS*, intralamellar septum; *LY*, lysosomes; *NU*, nucleus; *VA*, vacuole.

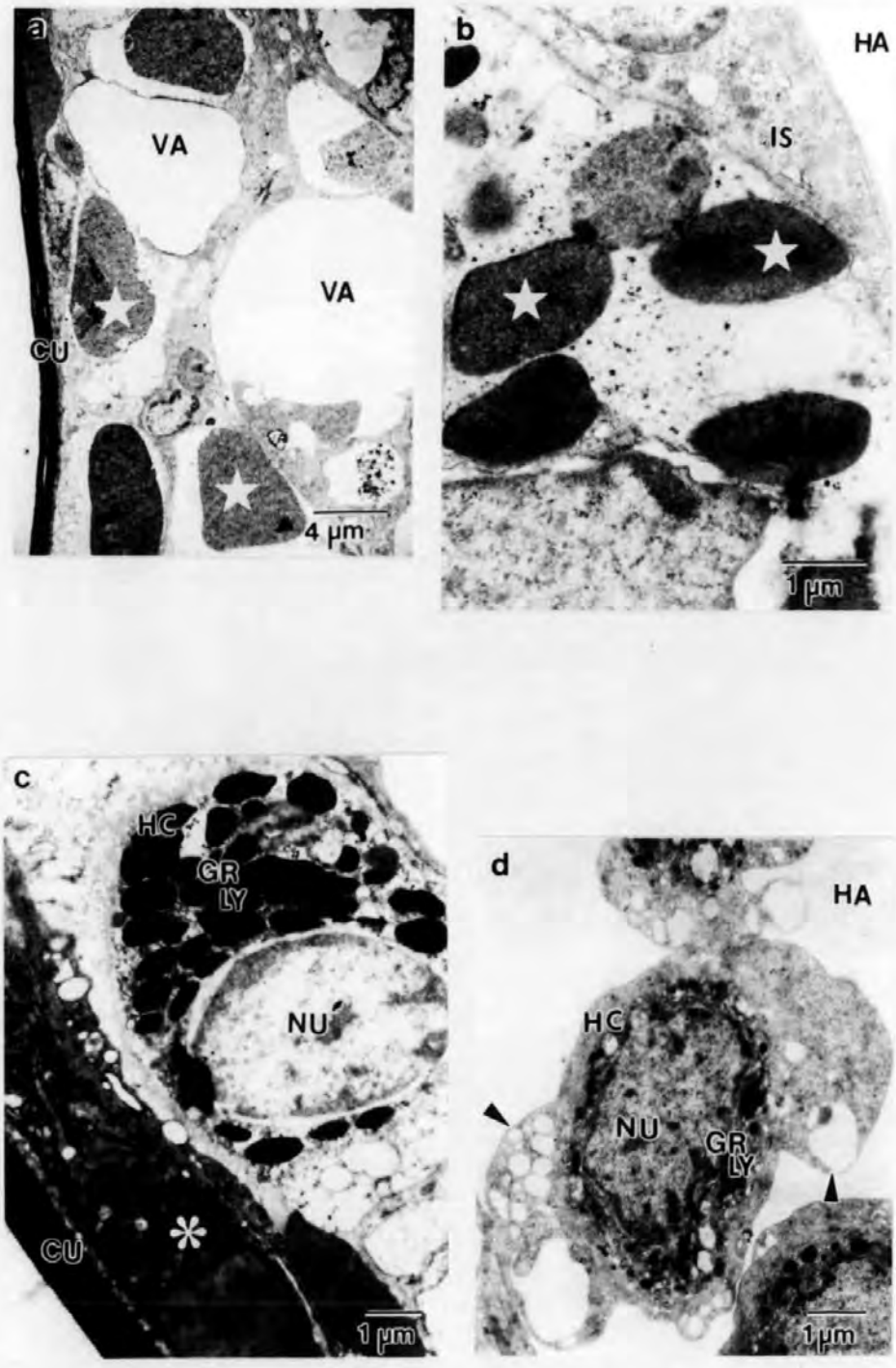
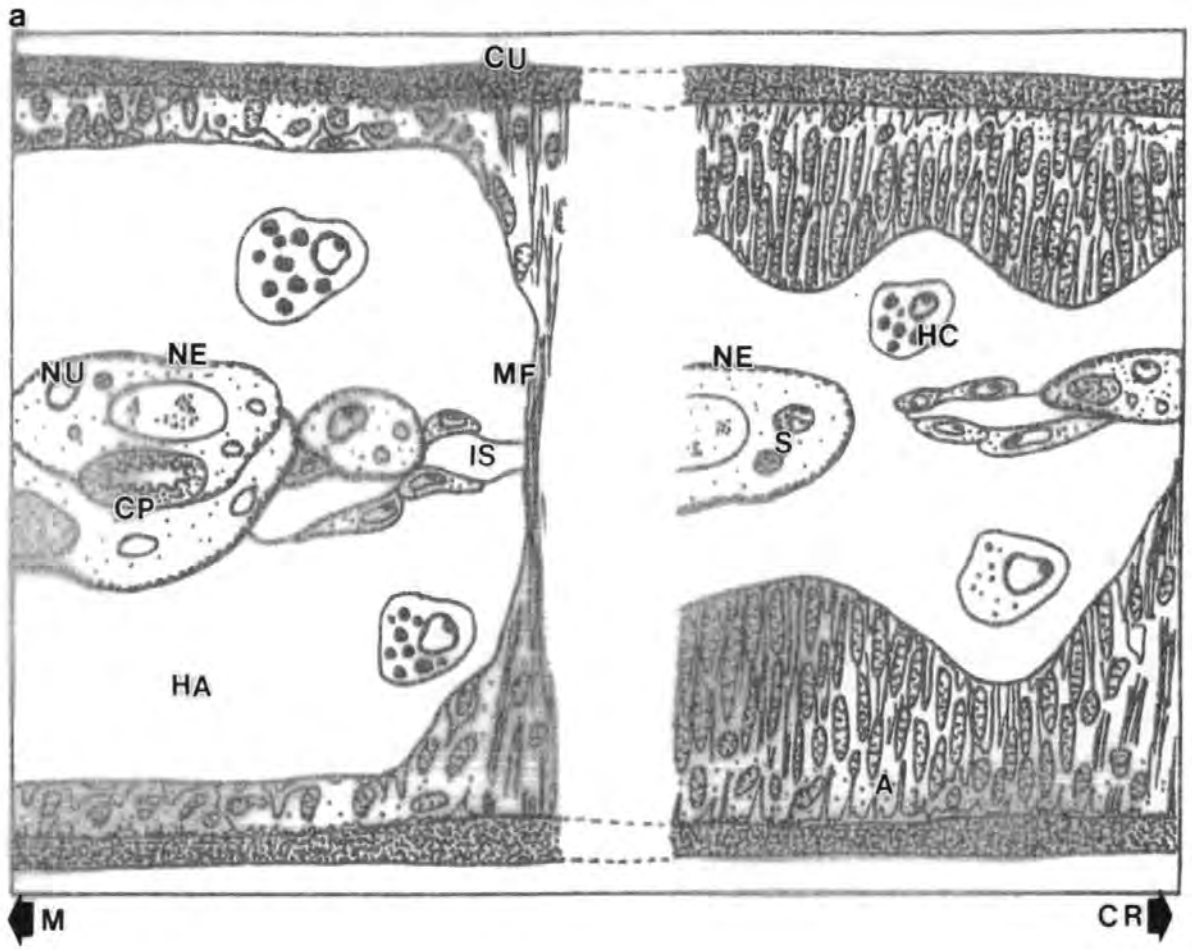

a
VA
VA
CU
4 µm
b
HA
IS
1 µm
c
HC
GR
LY
NU
CU
1 µm
d
HA
HC
NU
GR
LY
1 µm

Figure 2.10: a. Epithelial ultrastructure of a control posterior (No. 8) gill of *Carcinus maenas* proximal and distal to the marginal canal (M) [i.e. towards the central raphe (CR)]. **b.** Summary of the effects of exposure to 500 μg Cu L^{-1} on posterior gill epithelia proximal and distal to the marginal canal (M) [i.e. towards the central raphe (CR)], including epithelial thickening and haemocyte infiltration, apical and mitochondrial vacuolation, necrosis (∗) and lipofuscin granules, increased numbers of haemocytes and nephrocytes and subsequent blockage of the haemolymph channel (haemocoel). *A*, apical plasmalemma; *CP*, capillary; *CR*, central raphe; *CU*, cuticle; *CV*, central vacuole; *GR*, granules; *HA*, haemocoel; *HC*, haemocyte; *IS*, intralamellar septum; *LF*, lipofuscin granule; *LY*, lysosomes; *M*, marginal canal; *MI*, mitochondria; *NE*, nephrocyte; *NU*, nucleus; *S*, satellite vacuole; *VA*, vacuole. (not to scale)


a
CU
NU
NE
MF
IS
CP
HA
HC
NE
S
A
M
CR

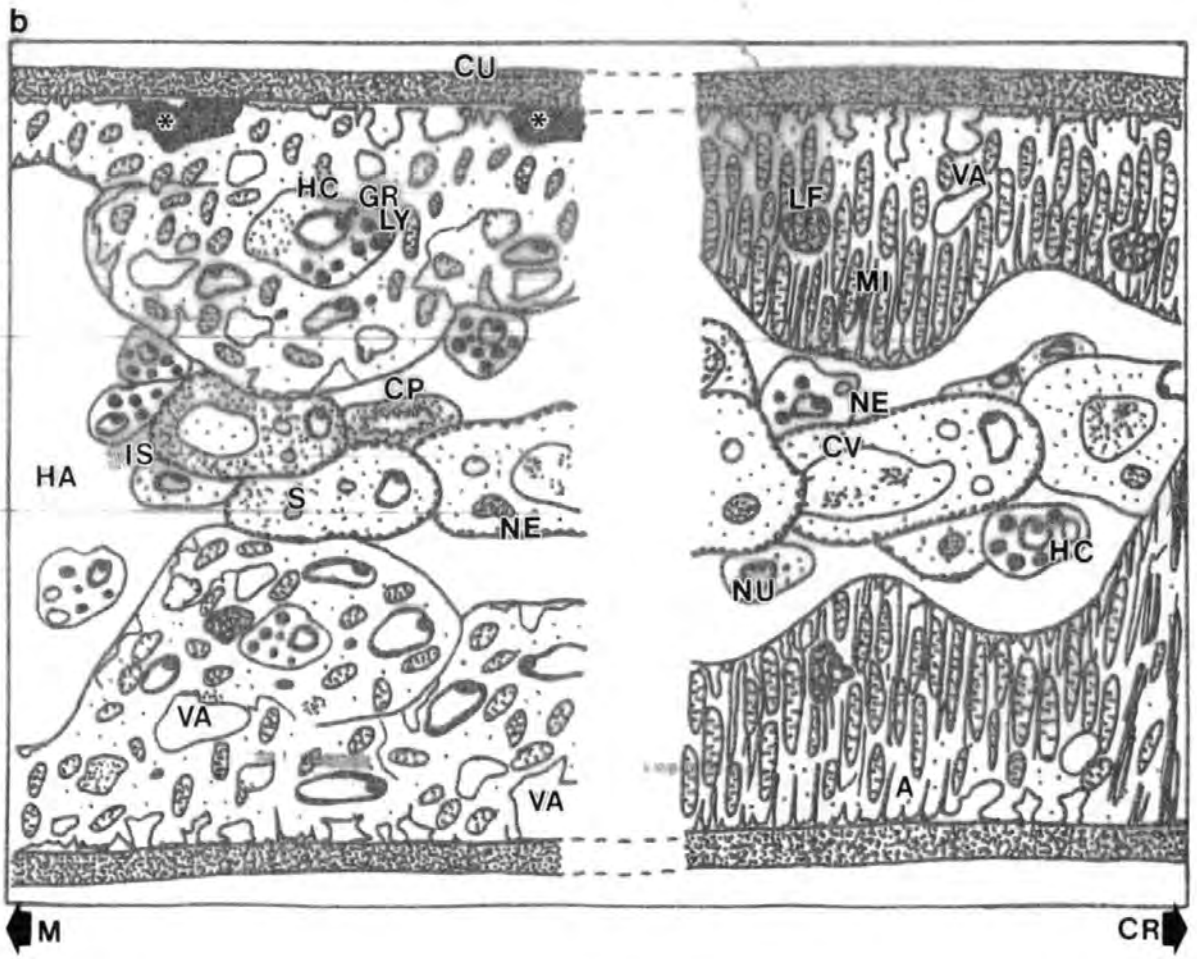
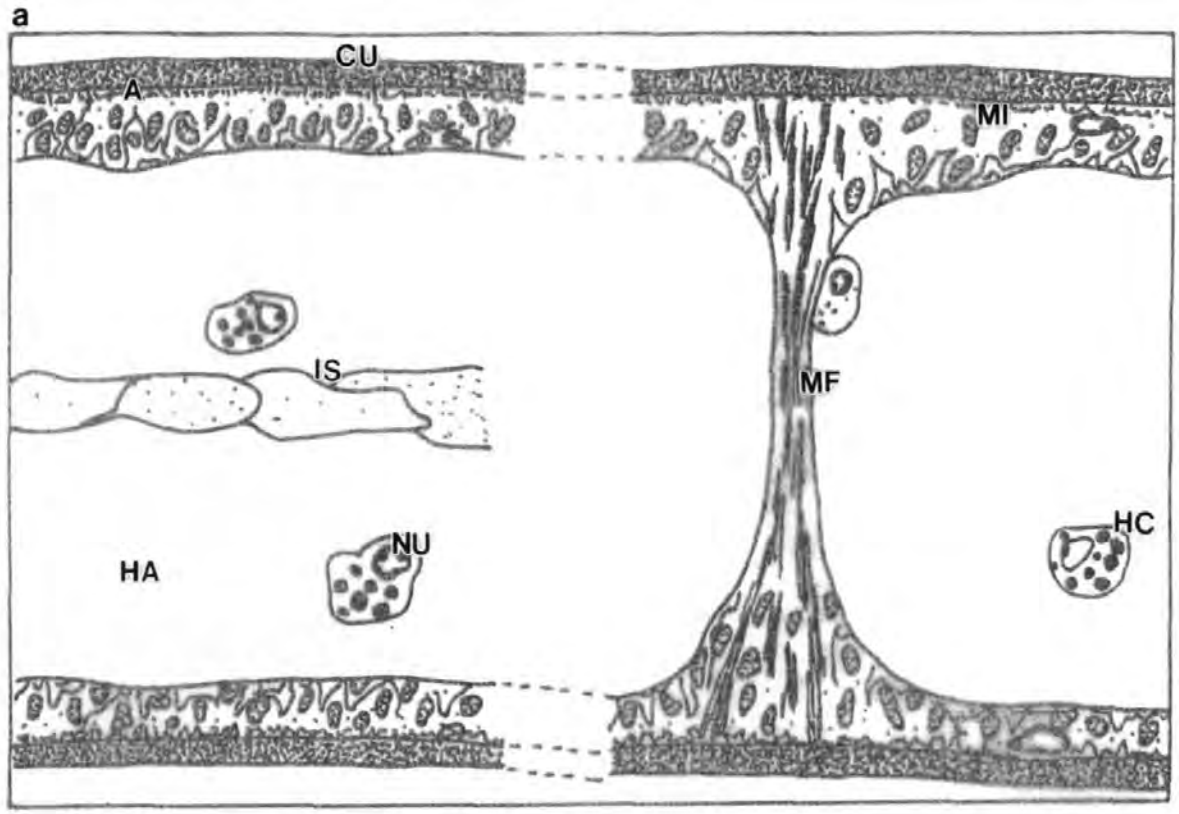
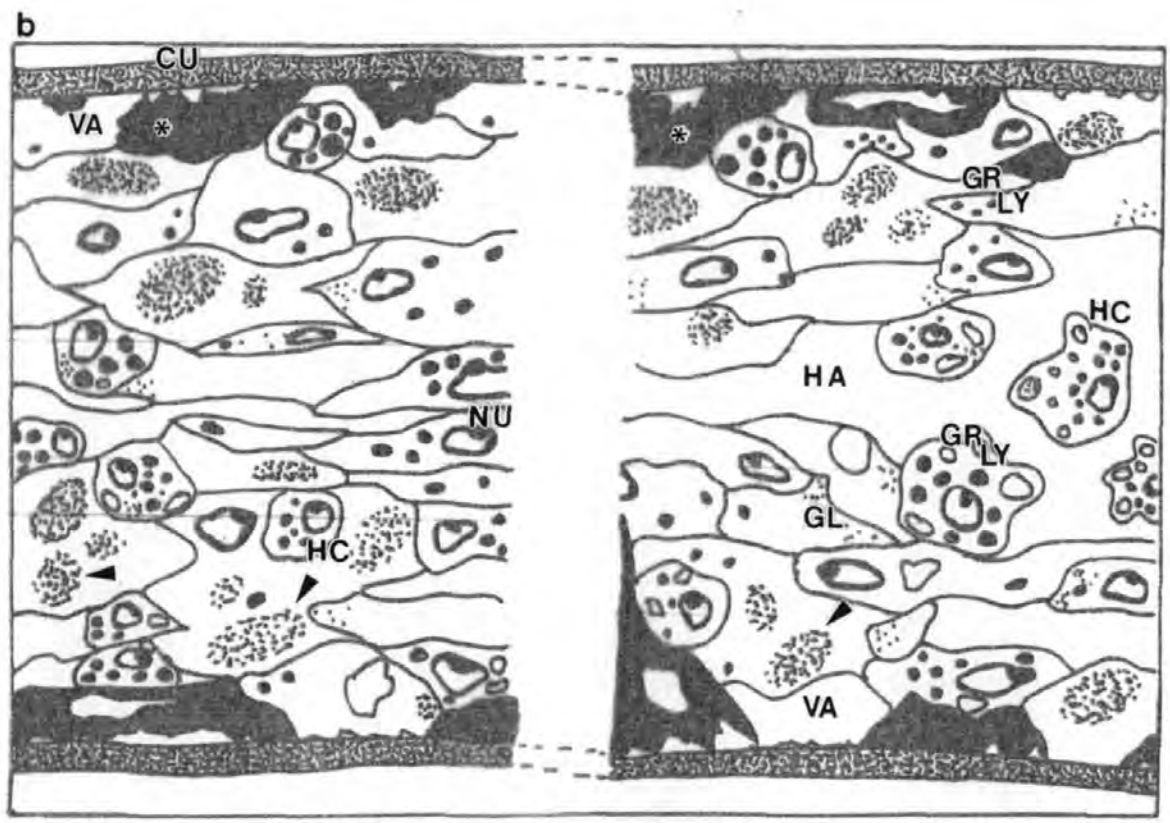

b
CU
HC
GR
LY
CP
IS
HA
S
NE
VA
VA
LF
MI
NE
CV
HC
NU
A
M
CR

Figure 2.11: a. Epithelial ultrastructure of a control anterior (No. 5) gill of *Carcinus maenas* proximal and distal to the marginal canal (M) [i.e. towards the central raphe (CR)]. **b.** Summary of the effects of exposure to 500 μg Cu L^{-1} on anterior gill epithelia proximal and distal to the marginal canal (M) [i.e. towards the central raphe (CR)], including epithelial hyperplasia and increased numbers of haemocytes with subsequent blockage of the haemolymph channel (haemocoel), vacuolation of haemocyte cytosol, severe necrosis (∗) and apical vacuolation leading to epithelial detachment, ovoid darkly-staining accumulations (arrow heads), free lysosomes/granules and glycogen. *A*, apical plasmalemma; *CR*, central raphe; *CU*, cuticle; *GL*, glycogen; *GR*, granules; *HA*, haemocoel; *HC*, haemocyte; *IS*, intralamellar septum; *LY*, lysosomes; *M*, marginal canal; *MF*, microfilaments; *MI*, mitochondria; *NU*, nucleus; *VA*, vacuole. (not to scale)


a
CU
A
MI
IS
MF
HC
NU
HA


b
CU
VA
GR
LY
HC
HA
NU
GR
LY
GL
HC
VA

CHAPTER 3

DOES OSMOREGULATORY GILL TISSUE ULTRASTRUCTURE IN *CARCINUS MAENAS* (CRUSTACEA, DECAPODA) REFLECT LOW-LEVEL COPPER EXPOSURE?

Abstract

Exposure of the common shore crab, *Carcinus maenas,* to 50 μg Cu L^{-1} in a flow-through seawater system did not result in consistent changes in cellular structure of the osmoregulatory gill tissue. Both control and copper-exposed gill epithelia exhibited great variability in cell condition, including severely vacuolated cells as well as cells without any vacuolation. Exposure to copper did not affect the frequency or structure of haemocytes and nephrocytes. These observations were also made in tissue samples processed immediately after field collection, after acclimation in the flow-through system, and during a 10 day recovery period. The observed variability in gill tissue appearance highlights the importance of adopting a morphometric approach to comparative ultrastructural studies at low exposure levels. Such an approach must include the quantitative analysis of cellular features from a statistically acceptable number of samples, to allow an objective assessment of tissue changes.

3.1 Introduction

Copper is a trace metal essential to all living systems, especially in its role as an enzyme co-factor (Harris, 1991). Copper is, therefore, beneficial at low levels in the environment, and indeed, a certain level is required to avoid symptoms of copper deficiency, which in humans can entail impaired growth, osteoporosis and degeneration of the central nervous system (Harris, 1991). As well as being essential to vertebrate metabolism, invertebrate aquatic crustaceans rely on the presence of copper within the tissues to guarantee the successful functioning of enzyme systems, and it is especially important as part of the haemolymph oxygen-binding pigment, haemocyanin.

One of the first organs to be impacted by exposure to water-borne metals are the gills. In general, experimental exposure of crustaceans to metals has involved relatively high concentrations (often measured in mg per litre) (e.g. Price and Uglow, 1980; Papathanassiou and King, 1983; Depledge, 1984a; Papathanassiou, 1985; see also Table 2.1), resulting in marked epithelial degeneration (*cf.* Chapter 2). An exception, however, is the study by Lawson *et al.* (1995), who exposed the common shore crab *Carcinus maenas* (L.) to 50 μg Cu L^{-1} in a flow-through seawater system. Such a level of copper was considered to be "environmentally realistic" by the authors and levels of total water-borne copper have recently been found to exceed this level in the Fal Estuary, even though mining activities in the area had ceased some years prior to the reported measurements (Hubert, 1995).

The ability of gill ultrastructure to recover from copper-induced damage, either during continued exposure or on re-introduction of clean conditions, has so far not been investigated satisfactorily. Nonnotte *et al.* (1993) observed such a recovery of the gill epithelia during prolonged exposure of *C. maenas* to 500 μg Cu L^{-1}, and Spicer and Weber (1992) have suggested that structural gill recovery may be the cause for a physiological recovery observed during metal exposure in *Cancer pagurus*. In the former case, however, it could not be confirmed if structural recovery occurred due to depletion of the metal levels in the static seawater system or during continued exposure to the initial exposure level.

The current study set out to assess the scope for ultrastructural recovery in the gills of *C. maenas*, following acute exposure (10 days) to copper at an environmentally realistic level (50 μg Cu L^{-1}) in a flow-through seawater system.

3.2 Materials and Methods

3.2.1 Collection of animals

In June 1995, intermoult male specimens of *Carcinus maenas* with green carapaces, and of approximately equal size (*c.* 6 cm) and weight (50-60 g wet weight) were collected from the Avon Estuary at Bantham (Devon) (Fig. 2.1), using drop nets baited with various species of fish. The animals were transported to the University of Plymouth aquarium where they were kept in aerated holding tanks containing filtered seawater (salinity of 35, 15°C) for 48 h, in a 12 h light/12 h dark cycle. Afterwards, crabs were placed in buckets of seawater and transported to the Brixham Environmental Laboratory (ZENECA Limited) and placed in a flow-through system.

3.2.2 The flow-through system

The experimental system consisted of 4 identical tanks (100 x 70 cm), each subdivided into 15 compartments of equal size (*c.*20 x 13 cm); the partitioning walls between compartments were perforated to allow the through-flow of seawater (salinity of 35) (Figs 3.1a and b). The seawater entered and left the system at an average flow rate of 1600 ml min^{-1}. The seawater was maintained 12.5°C (±0.2), oxygen levels ranged from 6.4 to 8.7 mg L^{-1} and pH values were constant at 8.15. The seawater was monitored continuously. Fourteen crabs were maintained in each copper-exposure tank and 7 in each of the control tanks, each animal being confined to a single compartment. Crabs were left to acclimate for one week prior to copper exposure. During acclimation, they were fed with chopped coley [*Pollachius virens* (L.)] at 3 day intervals before and after the exposure period (i.e. during "recovery"). The tanks were cleaned regularly by siphoning prior to exposure, during exposure and during the recovery period to avoid changes in water parameters and copper levels due to accumulation of metabolic wastes. Two of the tanks were exposed to sublethal levels of copper (50 μg L^{-1}) over a period of 10 days following the acclimation period. This exposure concentration remained constant due to continuous administration of the stock solution ($Cu_2Cl.2H_2O$ in water) with the seawater influx using a Watson Marlow Mhre 22 flow inducer (Fig. 3.2). The stock solution was renewed after 7 days. Following the 10 day exposure period, all tanks, including control tanks, were emptied by siphoning and allowed to refill with clean seawater. Flow conditions during the recovery period (10 days) were as described above.

3.2.3 Ultrastructural gill analysis

Five crabs were dissected immediately following their collection from the shore. In addition, one crab from each exposure tank, and a single animal from either of the control

tanks, was removed for dissection at each of the following times: following acclimation (7 days), exposure (10 days), and at 6, 12, 24 h, 2, 3, 4, 7 and 10 days during recovery. The remaining animals (after 27 days) were released back to the environment. Posterior gill number 8 was excised from the right gill chamber of each crab by cutting at the base. Gill tissue was processed as described in Section 2.2.2, however, following secondary fixation with osmium tetroxide, the tissue pieces remained in cacodylate buffer for up to 24 h before dehydration.

3.3 Results

Ultrastructure of the posterior gills of *C. maenas* did not vary between control animals (field, post-acclimation, experimental) and crabs that had been exposed to 50 μg Cu L^{-1} for 10 days. Similarly, gill tissue from animals sampled at intervals during the recovery period did not show any change with time.

In general, the gill ultrastructure corresponded to that described in Section 1.3.1, although mitochondrial and apical vacuolation of the epithelia was more frequent. Variability in the appearance of the epithelial cells was considerable, either showing a non-vacuolated ultrastructure, or containing numerous apical and mitochondrial vacuoles. This was true for all epithelial cell types present (i.e. chief, striated and pillar cells) and at all levels of treatment (i.e. following acclimation, control and exposure treatments as well as during "recovery") (Figs 3.3a to f; 3.4a to d). Haemocytes and nephrocytes were present throughout the lamellae, without apparent changes in their frequency or structure (Figs 3.4e and f).

3.4 Discussion

Exposure of *C. maenas* to an environmentally realistic level of copper in a flow-through seawater system did not result in any change in gill ultrastructure, although a cellular response has been reported previously, using the same species and experimental set-up (Lawson *et al.*, 1995). These ultrastructural changes, following a 10 day exposure period, included considerable epithelial vacuolation and an increase in the number of haemocytes within the gill lamellae (Lawson *et al.*, 1995). A possible reason for this different response may be related to the source of the crabs. Although the gill structure of control crabs used in the present study appears to be identical between the populations and those described by Lawson *et al.* (1994, 1995), the experimental crabs used by Lawson *et al.* (1995) were collected from a marine site, whereas the animals in the current study were taken from an

estuarine population. The latter population is adapted to the tidal changes in salinity encountered in the estuarine environment (Section 2.2.1). As the epithelial cells will be functionally adapted to these euryhaline conditions, and osmotic regulation and metal transport at least in part share the same mechanisms (Clausen *et al.*, 1993; Rainbow *et al.*, 1993; Rainbow, 1997a and b), this adaptation may infer greater tolerance to water-borne metal ions.

Geographically separated populations of *C. maenas* have been shown to differ in terms of their oxygen consumption (Aldrich, 1983) and trace metal accumulation rates (Chan *et al.*, 1992). In the latter study, crabs from a high salinity environment were shown to accumulate both zinc and cadmium at a significantly faster rate than animals adapted to a low salinity environment, when exposure took place in seawater of relatively high salinity (33) (Chan *et al.*, 1992). This corresponds to the greater sensitivity of experimental crabs used by Lawson *et al.* (1995) compared with crabs used in the present study. In general, the ability of estuarine organisms to tolerate continuously changing environmental variables is thought to equip these animals with an increased tolerance to trace metal exposure (Depledge, 1990b).

Another factor which may account for the different response of crabs used in the present study and by Lawson *et al.* (1995) is the difference in collection methods. The crabs used by Lawson *et al.* (1995) were sampled at low tide, by collecting those crabs that had remained stranded on the shore under rocks and within rock pools, whereas individuals for the present study were caught at high tide using baited nets. The "normal" behaviour of *C. maenas* is to move in with the rising tide and feed in the intertidal areas, returning to the lower shore with the receding tide. Those crabs remaining in the intertidal at low tide often lack one or more limbs (personal observation) and may be under a certain degree of physiological stress, even prior to experimental use [although Lawson *et al.* (1995) do not report the use of such individuals]. Sensitivity may vary further with size of the animals, and as those collected on the shore by Lawson *et al.* (1995) were smaller (*c.* 40 mm compared to *c.* 60 mm carapace width), the gills of these animals may be impacted at lower concentrations of trace metals than those of the larger specimens. Sample location, coupled with differences in animal size and condition, may, therefore, account for the varying degree of sensitivity to the level of copper applied in both sets of experiments. Differences in the water quality in the flow-through system, which may have lead to differences in the amount of bioavailable copper present (e.g. level of organics), can be excluded as an explanation. Measurements of water quality did not differ between

the periods of the present experiment and that carried out by Lawson *et al.* (1995) (Brixham Environmental Laboratory, pers. com.).

Variability between gill epithelial cell condition (i.e. degree of vacuolation) was far greater in the samples from this experiment than seen in any previous tissue samples (*cf.* Chapters 1 and 2). In fact, such variability has not been reported previously in the literature. Although the vacuolation observed is similar to mitochondrial swelling caused by poor fixation, in which the cristae become peripherally displaced (Bozzola and Russel, 1992), the fixation procedure used in the present study (Section 2.2.2) has been applied successfully in the past (*cf.* Chapter 2; Lawson, 1994). In addition, the fixation protocol includes osmotic adjustments of the chemicals involved to avoid such fixation problems as well as the use of very small tissue pieces (1 mm diameter) to avoid preferential fixation of marginal areas. The fixation protocol (Section 2.2.2) was modified slightly as the tissue samples had to remain in buffer following secondary fixation for up to 24 h to allow the transport of samples between laboratories. It is unlikely, however, that any changes to the tissue would occur following fixation.

In general, the results indicate that there is much variability in cell appearance in the gills of *C. maenas.* This finding has important implications for the study of low-level exposure effects on these tissues, as such variability will make it difficult to discern between natural variations in the degree of vacuolation and changes induced by exposure to metals. A much greater number of experimental animals and, therefore, tissue samples, would be necessary to make a definite statement about any damaging effect the copper may be exerting on the gill tissue, i.e. by morphometric analysis which would allow a statistical assessment of the existence and significance of tissue changes. A short-coming of the present study [also of Lawson *et al.* (1995)], is the relatively small number of experimental animals, due to restrictions in space and requirements for processing time (as well as the wish to conserve the population of *C. maenas* at the sample site). This, therefore, restricts the present study to a qualitative (rather than quantitative) assessment. At higher concentrations, however, where changes in cell ultrastructure are clear, or in non-comparative studies, qualitative assessments are sufficient. Indeed, in the former case statistical analysis may become impossible due to the complete disintegration of cellular features (*cf.* Section 2.3.5).

There was no ultrastructural gill response following static tank exposure (Chapter 2) of *C. maenas* to 50 μg Cu L^{-1}. Static tank exposure of animals from the Avon population showed that the ultrastructure of the posterior gills was not impacted until

300 μg Cu L^{-1}. Even at this exposure concentration, the damage observed was restricted to areas near the marginal canal (Section 2.3.4). There is some indication that metal exposure in a flow-through system leads to greater effects on crustaceans than exposure in a static system (Vernberg *et al.*, 1977). It seems unlikely, therefore, that low-level exposure effects are present and are being masked by the variability in tissue condition reported here.

The lack of any obvious structural damage to the gills following exposure to environmentally realistic levels is not surprising, as copper is an essential trace metal in the physiology of *C. maenas*. Internal mechanisms effecting the homeostatic balance of trace metals will be able to act in a detoxifying function, e.g. by binding to existing metallothionein, *de novo* synthesis of such metal-binding proteins and the transfer of copper to excretable granules (Viarengo and Nott, 1993). Compared with the crabs used by Lawson *et al.* (1995), a degree of tolerance to trace metal exposure may have evolved in the individuals from the Avon Estuary, accounting for the lack of response (Depledge, 1990b).

In summary, although this study set out to determine the ability of crab gill ultrastructure to recover from the effects of environmentally realistic exposure levels of copper, the chosen exposure duration and concentration failed to cause cellular damage. This lack of response compared with previous studies may be attributed to differences in the populations sampled, and to differences in collection techniques and animal size. Variability in the appearance of epithelial cells was considerable, ranging from highly vacuolated cells to cells lacking any vacuolation. This variability in cell appearance highlights the importance of large sample numbers to allow an objective morphometric assessment of cell condition to be made.

Figure 3.1: a. General view of the flow-through seawater system. **b.** Experimental perspex tanks, with in-flow (If) and out-flow (Of) routes indicated (arrows), showing individual compartments containing *Carcinus maenas*, and perforated dividing walls (Di).


a


b
If
Of
Di

Figure 3.2: Regulation of the constant addition of copper into the exposure flow-through tanks. *Cu*, copper stock solution ($Cu_2Cl.2H_2O$ in water); *Dc*, distribution chamber; *Fi*, flow inducer; *If*, in-flow to tanks; *Mx*, seawater and stock solution mixing cylinder; *Sf*, seawater flow.

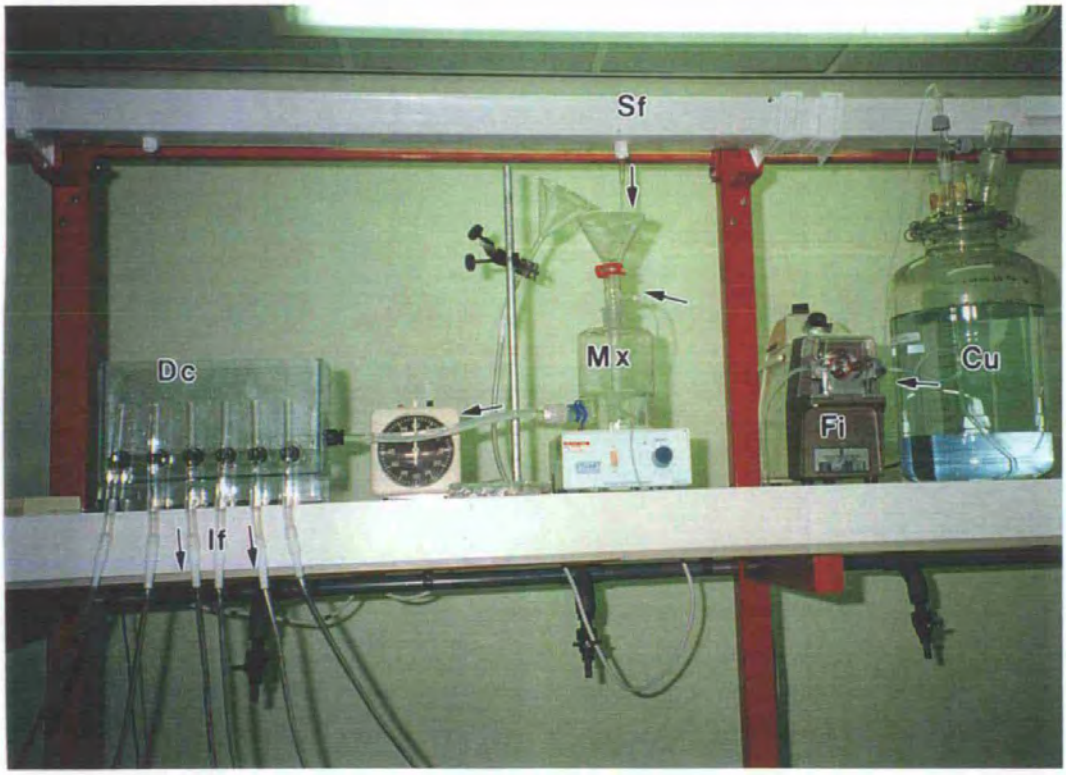
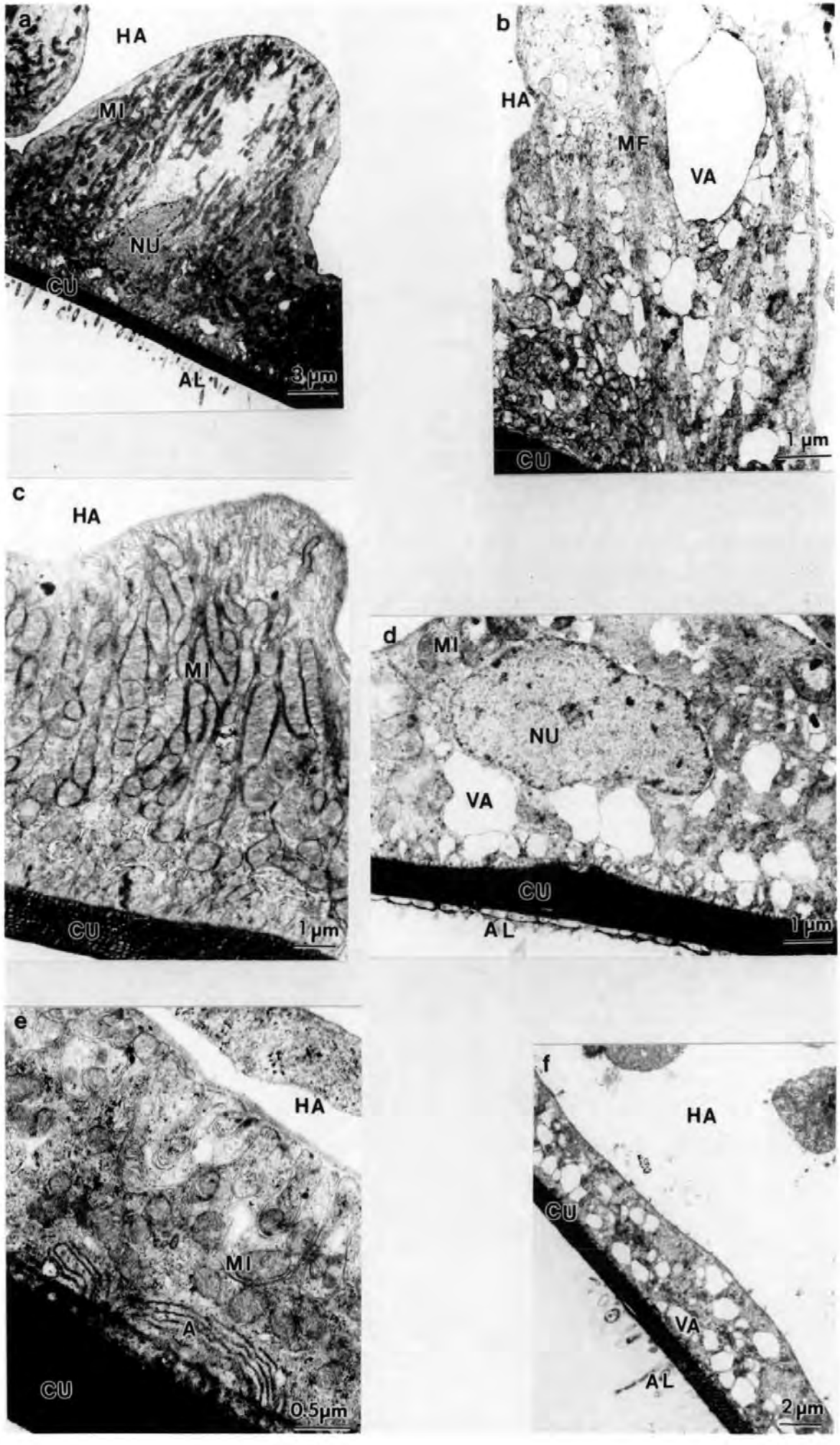

Sf
Mx
Dc
Cu
Fi
If

Figure 3.3: Epithelial cells in a posterior gill of *Carcinus maenas* following exposure to 50 μg Cu L^{-1} in a seawater flow-through system for 10 days, showing the variability in cell condition encountered. **a.** Non-vacuolated pillar cell. **b.** Severely vacuolated pillar cell. **c.** Non-vacuolated striated cell. **d.** Severely vacuolated striated cell. **e.** Non-vacuolated chief cell. **f.** Severely vacuolated chief cell. *A*, apical plasmalemma; *AL*, algal/bacterial layer; *CU*, cuticle; *HA*, haemocoel; *MF*, microfilaments; *MI*, mitochondria; *NU*, nucleus; *VA*, vacuole.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e
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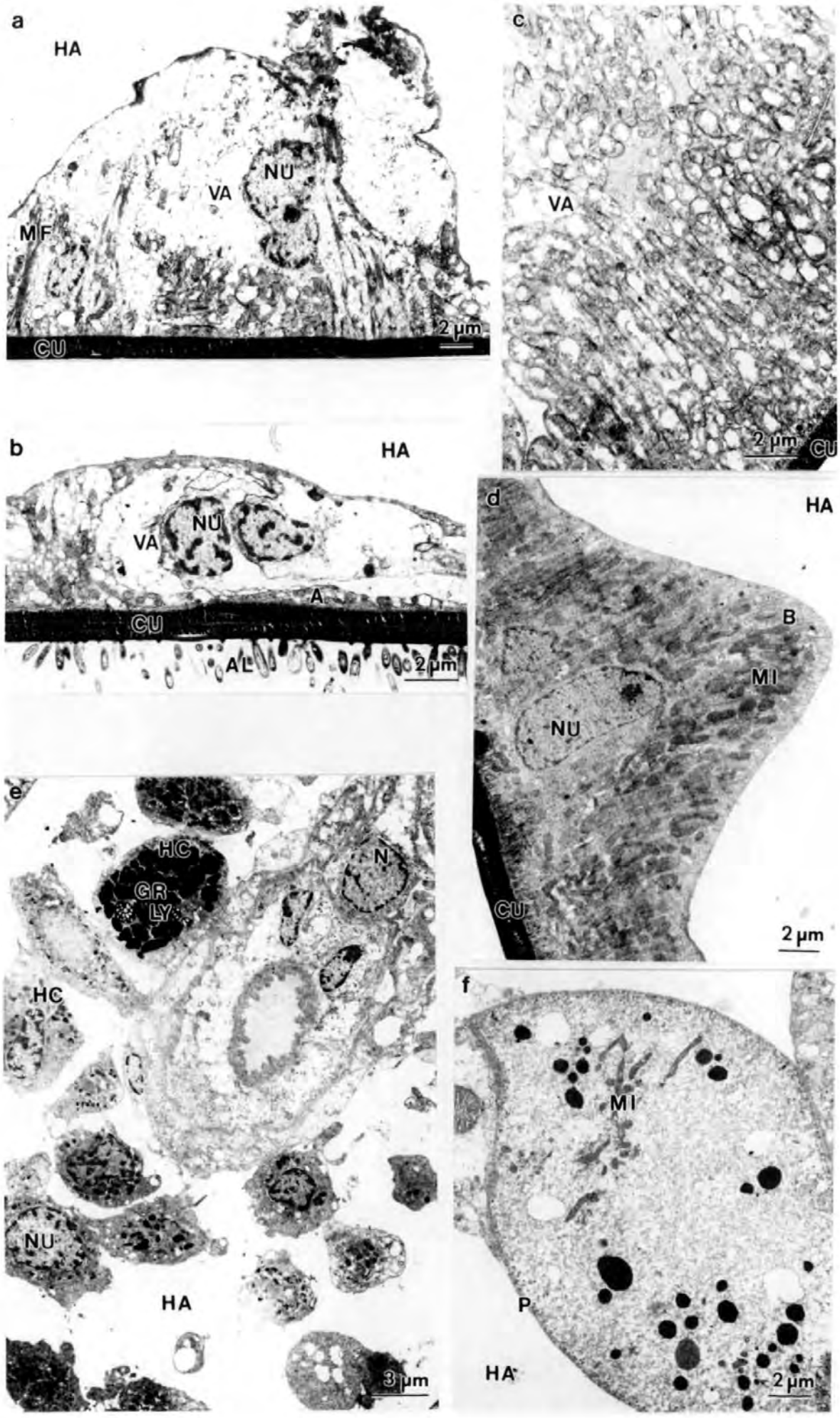
A
CU
0.5µm
f
HA
CU
VA
AL
2 µm

Figure 3.4: Cellular ultrastructure of posterior gills of *Carcinus maenas*. **a.** Severely vacuolated pillar cell in the posterior gill of a control animal. **b.** Severely vacuolated chief cell in the posterior gill of a control animal. **c.** Vacuolated epithelial cell after 10 days "recovery" from exposure. **d.** Non-vacuolated epithelial cell after 10 days "recovery" from exposure. **e.** Haemocytes in a posterior gill lamella of a crab exposed to 50 μg Cu L^{-1} in a seawater flow-through system for 10 days. **f.** Nephrocyte in a posterior gill lamella of a crab exposed to 50 μg Cu L^{-1} in a seawater flow-through system for 10 days. *A*, apical plasmalemma; *AL*, algal/bacterial layer; *B*, basal plasmalemma; *CU*, cuticle; *GR*, granules; *HA*, haemocoel; *HC*, haemocyte; *LY*, lysosomes; *MF*, microfilaments; *MI*, mitochondria; *NU*, nucleus; *P*, pedicels; *VA*, vacuole.


a
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CHAPTER 4

EFFECTS OF COPPER ON *CARCINUS MAENAS*: LINKING GILL ULTRASTRUCTURE, METALLOTHIONEIN CONCENTRATIONS AND SCAPHOGNATHITE ACTIVITY

Abstract

The use of the Computer-Aided Physiological Monitor (CAPMON) for the recording of scaphognathite activity was validated by comparing recordings made simultaneously from the same scaphognathite by CAPMON and a pressure transducer. Beat rates and patterns recorded were not significantly different, confirming the ability of CAPMON to detect scaphognathite movements.

Exposure of *Carcinus maenas* to 500 μg Cu L^{-1} for 10 days in a static system lead to overt ultrastructural damage within the gills. The respiratory gills were especially damaged and showed necrosis, severe vacuolation and blockages of the haemolymph space. Metallothionein concentrations within the gill tissue of copper-exposed crabs were not significantly different from levels in control tissue. In the copper-exposed animals, scaphognathite beat rate (recorded by CAPMON) was significantly decreased compared with control crabs. The total number of scaphognathite beats recorded in one hour was higher in copper-exposed than control animals. The increase was caused by a more equal relationship between left and right scaphognathites, more time spent in bilateral ventilatory activity and less time in both unilateral and bilateral apnoea.

Copper toxicity appears to be reflected in structural gill damage and physiological change, but not in metallothionein concentrations.

4.1 Introduction

Although many of the effects of heavy metals on crustaceans have been extensively reported (e.g. Bubel, 1976; Couch, 1977; Depledge, 1984a; Engel and Brouwer, 1984; Krishnaja *et al.*, 1987; Johnson, 1988; Hansen *et al.*, 1992a), there are very few studies relating morphological gill tissue changes to changes at other biological levels (but see Nonnotte *et al.*, 1993). In the previous chapter, the ultrastructural gill damage in *Carcinus maenas* caused by various sublethal concentrations of copper was described, and discussed in relation to functional implications and haemolymph parameter measurements made by other workers (Boitel, 1990). In this chapter, the ultrastructural gill damage following exposure to copper (500 $\mu g\ L^{-1}$) is related directly to changes at the molecular and physiological level. Three measurements were performed on the same organisms to observe an integrated response to trace metal exposure at different levels of biological organisation in *C. maenas*.

The molecular measurement involved quantification of metallothionein within the gill tissue of control and copper-exposed crabs. Metallothioneins are cysteine-rich, low molecular weight (6-10 kDa) proteins which play a major role in the detoxification of excess amounts of essential metals and non-essential metals (e.g. cadmium), and are normal constituents of the metabolism of metals within crustacean tissues, acting as storage sites for essential metals (copper, zinc) (Viarengo and Nott, 1993). Initially, binding of metal ions is achieved by displacement of metals (e.g. zinc) already bound to the protein, but ultimately *de novo* synthesis of metallothionein is initiated. In decapods, metallothionein concentrations vary according to moult stage and highest concentrations have been measured in midgut gland tissue of intermoult crabs (as used in the present study) (Engel, 1987). Metallothionein concentrations in the gill tissue of *C. maenas* are elevated by exposure to trace metals in the field (Pedersen *et al.*, 1997) and there are numerous reports of metallothionein induction in decapod crustaceans (e.g. Olafson *et al.*, 1979; Engel and Brouwer, 1984). Metallothionein, therefore, represents an appropriate molecular biomarker for the effect of experimental copper exposure.

The physiological measurement was scaphognathite activity. The beating of the scaphognathite, effected by the sequential levation and depression of muscles, is responsible for the flow of water through the gill chamber, allowing the exchange of ions and gases at the gill surfaces. At times, a change in the sequence of scaphognathite muscle contraction reverses the flow of water through the gill chamber. The present study addressed the hypothesis that dysfunctional gill epithelia may lead to compensatory responses in

scaphognathite activity as a result of tissue and haemolymph hypoxia of the gills caused by impairment of gaseous exchange processes (Nonnotte *et al.*, 1993). This hypothesis has not been tested previously for *C. maenas*, although the effects of exposure to metals on ventilatory activity have been described for the edible crab *Cancer pagurus* (Spicer and Weber, 1992) and the prawn *Crangon crangon* (Price and Uglow, 1980). In *C. pagurus,* scaphognathite activity remained unchanged following exposure to copper or zinc (0.4 mg L^{-1}) (Spicer and Weber, 1992), whereas exposure to relatively high levels of trace metals resulted in an increase of scaphognathite rate in *C. crangon* (Price and Uglow, 1980). An increase in ventilatory and reversal ("coughing") frequency has been reported in fish in response to zinc (Hughes and Adeney, 1977). Other environmental variables, including ambient oxygen levels, salinity and temperature, affect ventilatory activity in aquatic crustaceans. For example, exposure to hypoxic conditions, or low salinity, caused an increase in the scaphognathite activity of *C. maenas* (Taylor, 1976; Taylor, 1977). A rise in temperature lead to an increase in scaphognathite beat rate, as well as changes in the frequency and duration of ventilatory reversals in *C. maenas* (Cumberlidge and Uglow, 1977b; Taylor and Wheatly, 1979).

In the past, measurements of scaphognathite activity have been carried out using either an impedance technique (Hoggarth and Trueman, 1967; Dyer and Uglow, 1977) or a pressure transducer (Hughes *et al.*, 1969; Taylor and Butler, 1978). These techniques require either the invasive attachment of electrodes within the gill chamber or the insertion of cannulae. Both methods are likely to affect physiological performance by imposing stress on the experimental animals. In this study, scaphognathite activity was measured with the Computer-Aided Physiological Monitor (CAPMON), which has been used successfully for monitoring cardiac activity in invertebrates (Depledge and Andersen, 1990; Depledge and Lundebye, 1996). CAPMON offers a non-invasive method to record accurately the ventilatory behaviour of the animal. A minimum amount of stress is thereby exerted on the crab, as the instrumentation is attached to the carapace externally, requiring only few seconds of handling time. As the instrumentation does not affect the movements of the animals (within the confines of the experimental tank), attachment of sensors can be carried out days before physiological monitoring, thus allowing a true "picture" of ventilatory activity to be obtained. Most animals have a dominant scaphognathite, leading to periods of unilateral ventilatory activity (and, therefore, unilateral apnoea), or at least to a higher mean beat rate of that scaphognathite (Arudpragasam and Naylor, 1964a, b). Generally, unilateral

bilateral apnoea, i.e. with both scaphognathites at rest (Arudpragasam and Naylor, 1964a, b).

This study set out to elucidate the links between ultrastructural gill damage, functional changes (ventilatory scaphognathite activity), and the onset of molecular detoxification mechanisms (metallothionein synthesis) in the shore crab *C. maenas*, following exposure to water-borne copper.

4.2 Materials and Methods

4.2.1 The Computer Aided Physiological Monitor (CAPMON)

The Computer Aided Physiological Monitor (CAPMON) was described in detail by Depledge and Andersen (1990). The system comprises sensors, a transducer/computer interface, and a PC (or laptop computer) for data display and recording (Fig. 4.1a). Movements of the scaphognathites and/or cardiac activity are detected by infra-red phototransducers attached to the carapace anteriorly above the scaphognathites or centrally above the heart (Fig. 4.1b). The sensors are attached by glueing plastic "collars" to the carapace, into which the sensors are introduced and held in place by small screws (Fig. 4.1b). These collars can be left attached indefinitely, thus minimising the actual handling time prior to physiological monitoring. Each sensor comprises an infra-red light emitting diode and a phototransistor detector. Both components are positioned parallel to each other, the photo transistor detecting variations in the reflected infra-red light intensity, caused by the passage of the scaphognathite within the gill chamber. The generated light-dependent current is passed on (via thin and highly flexible coated wires) to the transducer/computer interface. The circuitry is described by Depledge and Andersen (1990). Data are displayed in graphical form on the computer screen (4 sensors per PC) and recorded as rate measurements (beats per minute). The data files can be accessed by common spreadsheet/statistics packages. Initial data handling in this case was carried out using *Microsoft Excel for Windows (Version 5.0)*.

4.2.2 Verification of the ability of CAPMON to detect scaphognathite movement

As the CAPMON system has not been used previously to record scaphognathite beating, the technique was validated by comparing results from the same crab using CAPMON and a pressure transducer; the latter represents an established method for recording scaphognathite activity (e.g. Taylor and Butler, 1978). A crab (taken from Plymouth Sound, Devon) was fitted with two sensors, one placed above each scaphognathite, and allowed to settle in a bucket of seawater. Recordings of scaphognathite activity were made

over 30 min. During this time, the dominant (i.e. most active) scaphognathite was identified, and, therefore, chosen for the measurement of pressure changes using a physiological pressure transducer (Gould Statham, P23 Series). A hole was drilled into the carapace of the crab approximately above the midgill region of posterior gill number 6 (Taylor and Butler, 1978) (i.e. in a more posterior position than the CAPMON sensor already present). A cannula (size 6FG), primed with seawater, was inserted into the hole, reaching through the interior membrane into the gill chamber. The tubing was secured with super glue. The cannula was attached to a three-way tap to allow purging of the pressure transducer and thus avoid interference by enclosed air bubbles. The pressure transducer was held in place at the same level as the crab (the latter submerged in seawater in a glass beaker) and connected to a polygraph (GRASS, Model 7C), adjusted for minimum noise and maximum sensitivity. Scaphognathite movement was recorded as a trace expressing pressure changes and observed on the computer screen linked to the CAPMON, which recorded scaphognathite activity as beats per minute. Recordings made by the two techniques showed the same patterns and rates of activity, changes in pressure being recorded parallel with scaphognathite movements detected by the infra-red sensor. Figure 4.2 shows extracts from the trace recorded by the polygraph, every larger oscillation depicting a gradual beat of the scaphognathite also observed on the CAPMON computer (Fig. 4.2a). The more pronounced oscillations of the trace mirror a sudden increase in beat rate coupled with a scaphognathite reversal (Fig. 4.2b).The straight line on the polygraph plot represents a rest phase in scaphognathite movement, the pressure remaining unchanged (Fig. 4.2c). These characteristics were visible simultaneously on the CAPMON computer screen. Statistical analysis of beat rate recordings showed that there was no significant difference between the two recording techniques (One-way ANOVA; $F=0.18$, $p=0.6799$) (Fig. 4.3).

The simultaneous use of an established method for measuring scaphognathite activity within the gill chambers of *C. maenas* (the pressure transducer) and the Computer-Aided Physiological Monitor (CAPMON) confirmed that the infra-red CAPMON sensors were capable of detecting the beat movement of scaphognathites. Not only did the CAPMON system provide similar measurements of the number of beats by the scaphognathites, but also provided equal measures of ventilatory characteristics such as force of the scaphognathite beats (observed as differences in trace amplitude both on the computer screen and on the polygraph trace) and ventilatory apnoea (straight line trace). Verification of this non-invasive monitoring technique was carried out for the original

photoplethysmograph instrument (Depledge, 1984b), however, further verification was necessary as the CAPMON system had undergone various alterations and technical improvements [e.g. to eliminate the heat emission originally caused by the instrument (Depledge, pers. com.)]. Furthermore, the location chosen for the attachment of sensors for the recording of scaphognathite movements (on the dorsal surface to minimise stress to the animals and maximise detection of scaphognathite movements) did not correspond to that used by Depledge (1984b) (who attached sensors ventrally). The mean number of beats recorded by the polygraph trace was not significantly different from the CAPMON recordings.

CAPMON has the advantage over the pressure transducer of being a non-invasive monitoring technique not requiring insertion of cannulae into the carapace. The animal faces stress, therefore, only during attachment of the collars and sensors, however, collars can be attached several days preceding the start of an experiment. Attachment of the infra-red sensors imposes little stress on the animal and, subsequently, leaves the animal relatively uninhibited in terms of movement. Scaphognathite activity is less likely to be influenced by handling stress, invasive treatment or restraining, giving CAPMON a definite advantage over other monitoring techniques. Furthermore, CAPMON can be applied to animals in the field (Bloxham, pers. com.) and for long-term monitoring, making it an extremely valuable physiological monitoring tool. One failing of CAPMON as a recording technique of scaphognathite movement, however, was its inability to identify ventilatory current reversals, an integral characteristic of the ventilatory behaviour of *C. maenas* (Arudpragasam and Naylor, 1964a). These reversals result from a change in the excitation sequence of the depressor and levation muscles in the exopodite of the maxilla (scaphognathite) (Young, 1975). Originally, such reversals were thought to be important in the ventilation of the posterior gills (which are relatively distant from the ventilation current entering through the Milne-Edwards opening above the chelae) (Arudpragasam and Naylor, 1964a). Hughes *et al.* (1969) found that the pressure differentials in the posterior gill chamber were minor and suggested that ventilatory reversals were part of the gill cleansing process (effected mainly by the epipodite of the third maxilliped), as well as counteracting the formation of stagnant diffusion barriers. Reversals were identified during the simultaneous application of the two recording techniques used here and were visible on the CAPMON computer screen as sudden short-lived increases in beat rate (approximately 300 bpm instead of 60 bpm). In the resting ventilatory physiology of *C. maenas*, reversals account for merely < 8% of time spent in

ventilatory activity, with a frequency of 18 reversals per hour, each lasting for approximately 3 seconds (Cumberlidge and Uglow, 1977a), and are unlikely to have a significant effect on results. For the purpose of the following studies, it was concluded that the advantages of CAPMON as a non-invasive technique for recording scaphognathite movement far outweigh any disadvantage in not being able to detect beat reversals.

4.2.3 Collection and holding conditions

In January 1997, 32 individuals of *C. maenas* were collected from the Avon Estuary at Bantham, Devon (for detailed site description see Chapter 7), using drop nets baited with various species of fish. Only males with a green carapace, and of approximately the same weight (50-60 g) and carapace width (6 cm) were taken. The animals were transported to the Plymouth University aquarium in buckets containing seawater (salinity of 35), and kept in a seawater holding tank (salinity of 35; water temperature of 15°C; 12 h dark/12 h light) for 5 days before being transferred into the experimental tanks. Crabs were fed with gamma-irradiated mussel (*Mytilus edulis*) on the day following collection and on the day prior to commencement of the experiment. The experimental tanks were glass, and had been acid-washed previously and filled with the appropriate experimental solution (full seawater, or full seawater containing 500 μg Cu L^{-1}, added as a $Cu_2Cl.2H_2O$ stock solution) to a volume of 20 L for 4 days to allow absorption of metal to the walls. This pre-treatment minimised the loss of copper from solution during the 48 h between water changes. Water samples were taken immediately before and after a water change to determine if any copper depletion had occurred within each 48h period. Water samples were analysed by atomic absorption spectrophotometry (Varian SpectrAA 600). It was established that losses of copper over 48 h did not exceed 14%. Four crabs were placed in each experimental tank (total of 16 copper-exposed and 16 control animals) and left for 10 days.

4.2.4 CAPMON experimental procedure

All animals spent a minimum of 15 days in the aquarium (i.e. acclimation time plus time in experimental tanks) prior to physiological monitoring, allowing sufficient time for the disappearance of tidal ventilatory rhythms. After 9 days in the experimental tanks, animals were removed for the attachment of plastic collars (Section 4.2.1) and returned to the experimental tanks. On the following day, 4 animals were each removed from a control and an exposure tank, and fitted with two sensors, one above each scaphognathite (Fig. 4.1b). The crabs were placed individually in 2 L glass beakers, which had been covered

previously with black polythene and filled with the appropriate experimental medium. Polystyrene lids, through which aeration of the water was continued, were fitted. Water temperature was maintained at 15°C. The crabs were left for 1h to acclimate and regain resting ventilatory levels, during which adjustments at the transducer/computer interface regarding sensitivity and apparent "noise" were made. Data were recorded during the hour following acclimation. The scaphognathite activity of 16 control and 16 copper-exposed animals was recorded.

Where possible, the CAPMON data were analysed using parametric tests, however, when data did not meet the requirements for parametric analysis, non-parametric statistical analyses (e.g. Mann-Whitney; Kruskal-Wallis) were used. In the two experimental groups, scaphognathite activity was assessed in terms of the total scaphognathite beats during the 1 h monitoring period (One-way ANOVA). The medians of the CAPMON monitoring data of the two groups (using all data >0 bpm), equivalent to the median beat rate per minute, were compared using the Mann-Whitney Two-sample Comparison, the distribution of the data using the Kolmogorov-Smirnov Two-sample Test. Scaphognathite activity relationships were assessed in terms of the relative contributions of left and right scaphognathites to the scaphognathite beat total (One-way ANOVA), and by establishing the amount of time spent in bilateral ventilatory activity (i.e. both scaphognathites beating simultaneously), bilateral apnoea (i.e. neither scaphognathite active) as well as unilateral activity (i.e. either left or right scaphognathite active) (Kruskal-Wallis and Mann-Whitney Two-sample Comparisons). Periods of unilateral apnoea were compared between the copper-exposed and control animals with regard to the median number and length of ventilatory pauses occurring during the 1 h monitoring period (Kruskal-Wallis Two-sample Test).All statistical tests were performed using *Statgraphics Plus for Windows (Version 2.1)*.

4.2.5 Ultrastructural gill analysis

Following physiological monitoring, a posterior (number 8) and an anterior gill (number 5) were removed from the right gill chamber of two control and two copper-exposed crabs. Further tissue processing for electron microscopical analysis was carried out as described in Section 2.2.2.

4.2.6 Metallothionein quantification

Following physiological monitoring, the gill tissue not used for ultrastructural examination was removed for metallothionein (MT) quantification. The excised gills were frozen immediately in liquid nitrogen and kept at -80°C until further processing. The method used

for the quantification of MT follows the three-step process of Pedersen *et al.* (1994) (Fig. 4.4). The first step involved pre-treatment by homogenisation and ultracentrifugation. The tissue samples from each crab were ground individually in liquid nitrogen, to which 3 ml of 1 mM dithiothreiotol (DTT) and 30 µL of phenylmethylsulphonylfluoride (PMSF) solution were added, and the mixture sonicated (3x15 s) to release the cytosol from the cells. Another 30 µL of PMSF solution was added and the mixture ultracentrifuged for 70 min at 55000 rpm at 4°C. The supernatant was divided up into Eppendorf tubes by pipetting and frozen at -80°C before further processing. Partial purification of the supernatant fraction (cytosol) by solvent extraction (using ethanol rather than acetone) represented the second step, in which the supernatant was allowed to defrost at room temperature. Thawed supernatant (500 µL) was added to a centrifuge tube, together with 500 µL of absolute ethanol (at -20°C) and 40 µL of chloroform, and the mixture vortexed. After 10 min of centrifugation at 8000 rpm, the supernatant was transferred into a fresh centrifuge tube and 3ml of absolute ethanol added. These centrifuge tubes were allowed to cool at -20°C for 1 h before centrifugation was repeated. The resulting supernatant was discarded and the pellet washed with 2 ml of Tris-HCl washing buffer (pH 8). This was vortexed and centrifuged. The supernatant was discarded and the pellet put on ice and dried with argon and/or nitrogen gas. The dried pellet was dissolved in 300 µL of tris-ethylenediaminetetraacetic acid (EDTA) buffer (pH 7) by vortexing, and 4.2 ml of dithio-bis/2-nitrobenzoic acid (DTNB) added to the samples. After allowing the samples to react for 15 min in the light, the third step, MT quantification by measurement of the thiol groups, was carried out using a UV/Vis spectrophotometer (Unicam, UV4), reading absorbance at 412 nm wavelength and using glutathione (GSH) as a reference standard. Metallothionein measurements in copper-exposed and control gill tissue were compared by One-way ANOVA [*Statgraphics Plus for Windows (Version 2.1)*].

4.3 Results

4.3.1 Gill ultrastructure

Gill ultrastructure of the anterior and posterior gills taken from control and copper-exposed animals confirmed that 10 days of exposure to 500 µg Cu L^{-1} caused the same cellular differences between copper-exposed and control tissues as reported in Section 2.3.5 (see Figs 2.10 and 2.11 for a summary). Damage included total disorganisation of the anterior gill epithelia into a multilayered hyperplasic structure, containing many haemocytes or parts thereof (granules, lysosomes, nuclei). Necrotic areas, as well as large

vacuoles, were linked to the partial detachment of the epithelial layer from the cuticle. In general, the gill epithelial cytosol of copper-exposed crabs was not contained within the membrane compartments due to a complete breakdown of cellular structure. Endoplasmic reticula and free ribosomes were rarely discernible. Posterior gill epithelia showed greatest damage in the areas proximal to the marginal canals. In this region, epithelia were thickened, infiltrated by haemocytes and vacuolated. There were necrotic areas close in the apical cell regions, as well as lipofuscin granules in the more basal regions of the epithelia. In the areas distal to the marginal canal (towards the central raphe), exposure to copper caused the formation of lipofuscin granules and an increase in apical cell vacuolation. There appeared to be higher numbers of haemocytes present both in the anterior and posterior gills, as well as greater numbers of nephrocytes in the latter compared with control tissues.

4.3.2 Metallothionein

Although the gill tissue of copper-exposed crabs contained higher metallothionein (MT) concentrations than the gill tissue of control animals (Fig. 4.5), the difference was not significant (One-way ANOVA; $F=3.87$; $p=0.0588$).

4.3.3 Scaphognathite activity

There was no significant difference between the total scaphognathite beats recorded during the 1 h monitoring period between control and copper-exposed crabs (One-way ANOVA; $F=0.01$; $p=0.9439$) (Fig. 4.6). For both groups, the mean total number of beats in 1 h was *c.* 4500. The scaphognathite beat rate (beats per minute/bpm) (excluding apnoea periods), however, was significantly lower in copper-exposed than in control animals (Mann-Whitney Two-sample Comparison; $W=1.07$; $p=0.0116$) (Fig. 4.7). Figure 4.8 illustrates the frequency distribution of different beat rates for control and copper-exposed animals, including periods of ventilatory rest (i.e. 0 bpm). The results show that control animals spent more time at ventilatory rest than copper-exposed animals (Fig. 4.8). The latter, however, spent proportionally more time at beat rates up to 60 bpm, whereas higher beat rates were more common in control animals (Fig. 4.8). The Kolmogorov-Smirnov (K-S) Two-sample Test showed that data distributions of rates >0 bpm were significantly different (K-S statistic=2.05; $p=0.0005$), the maximum distance between the two cumulative frequency plots (DN) being 0.075 (Fig. 4.9). The data, presented further as a frequency distribution histogram, confirm that the control animals spent relatively more of their active ventilatory time at beat rates exceeding 60 bpm than the copper-exposed group (Fig. 4.10). The most common beat rate in copper-exposed animals was <60 bpm.

There were no significant differences (One-way ANOVA) between the contributions made to the total number of beats per hour by left and right scaphognathites in either control ($F=0.44$; $p=0.5123$) or copper-exposed animals ($F=0.02$; $p=0.8822$) (Fig. 4.11).

Activity relationships between scaphognathites (i.e. time spent with both, neither, or only one scaphognathite active) in control and copper-exposed animals were not significantly different (Table 4.1). Certain trends, however, were apparent. Exposed animals spent relatively more time in bilateral scaphognathite activity, and less time in bilateral apnoea, than control crabs (Fig. 4.12). In addition, the right scaphognathite was dominant in both groups (Fig. 4.12).

There were no significant differences (Kruskal-Wallis Two-sample Tests) in the number of periods of unilateral apnoea in the activity of any scaphognathite (test statistic=0.1; $p=0.7541$) or median length of pauses (test statistic=3.74; $p=0.053$) (Figs 4.13 and 4.14).

4.4 Discussion

Exposure of *C. maenas* to 500 μg Cu L^{-1} for 10 days resulted in the same ultrastructural gill degeneration as described following an exposure period of 14 days (Section 2.3.5). Respiratory gills were damaged throughout the lamellae, with cellular hyperplasia leading to blockages of the haemolymph space. Extreme necrosis, membrane disruption and vacuolation resulted in the detachment of the epithelial layer from the cuticle, suggesting severe impairment of gill function. Despite the excessive structural gill damage, concentrations of metallothionein were not significantly increased following the 10-day exposure period. Concentrations in the copper-exposed gill tissue, however, were elevated compared with control tissue, indicating that a degree of detoxification had occurred. Generally, in invertebrates, there is a lag in metallothionein induction following trace metal exposure (Roesijadi, 1982), with toxic effects being manifested in the gill tissue prior to the induction of metallothionein (Roesijadi, 1993). Severe cellular disruption, coupled with a lag in synthesis induction, may, therefore, explain the apparently weak metallothionein response in *C. maenas* following copper exposure reported here.

The total number of scaphognathite beats was not significantly different between control and copper-exposed animals. Interestingly, the median scaphognathite beat rate for each group (calculated under the exclusion of apnoea periods) was significantly decreased following exposure to copper. Although measurements of oxygen utilisation of ventilatory

currents were not conducted in the present study, Arudpragasam and Naylor (1964a) reported an increase in the percentage oxygen utilisation simultaneously with a decrease in ventilatory rate. Ventilatory rate changes in the current study may, therefore, represent a means of increasing uptake of oxygen from the ventilatory current by the damaged gill epithelia. The median beat rates for control and copper-exposed animals in the current study (46 bpm and 43 bpm respectively) correspond to the levels reported previously for *C. maenas* "at rest" (Cumberlidge and Uglow, 1977a), but are slightly lower than reported by Jouve-Duhamel and Truchot (1985). The decrease in median scaphognathite rate resulted from copper-exposed crabs spending a higher percentage of their active ventilatory time at beat rates <60 bpm. Control crabs were more active at beat rates >60 bpm. An increase in overall scaphognathite beat rate has been reported in *C. crangon* following a 48 h exposure to 100 μg Cu L^{-1} (Price and Uglow, 1980). Although the authors did not suggest any direct cause for this change, the increase in scaphognathite rate may have been related to damaged gill tissue and thus impaired gas transfer, causing hypoxia and subsequently a compensatory response in ventilatory activity to increase oxygen delivery to the gills. It is also possible that scaphognathite activity is altered due to chemosensory detection of the trace metal, or affected by the direct toxic action of copper on the nervous system (Waldichuk, 1974). The latter effect has been implicated as the reason for disturbances in ventilatory behaviour of *Palaemon adspersus* following exposure to sublethal concentrations of crude oil extract (Baden and Hagerman, 1981). It seems that there is no common crustacean scaphognathite beat rate response to metal exposure, and results indicate a dependency on exposure level, duration and, most importantly, species. Observations made in this study are, therefore, not easily applied to other experimental situations.

Analysis of the ventilatory activity of *C. maenas* showed that exposure to copper did not significantly affect the relative contributions made by the left and right scaphognathites to the total beats recorded. The dominance of either scaphognathite, a typical feature of the respiration of *C. maenas* (Cumberlidge and Uglow, 1977a), was less pronounced in the copper-exposed animals.

Although the above differences in scaphognathite activity of the two groups were not significant, the trend to beat bilaterally, rather than unilaterally or not at all, has implications for the total ventilation volume, as ventilation is related directly to scaphognathite beat frequency (Arudpragasam and Naylor, 1964a; Cumberlidge and Uglow, 1977b). Assuming that the gain from the increase in bilateral activity exceeds the

reduction caused by the drop in overall ventilatory rate, the increase in bilateral scaphognathite activity contributes to the rise in total ventilation volume. The decrease in the amount of time spent in unilateral activity by copper-exposed *C. maenas* implies that resting oxygen demands may not be met by ventilation of a single gill chamber. Indeed, exposure of *C. maenas* to 500 μg Cu L^{-1} and the resulting ultrastructural damage have been linked previously to haemolymph hypoxia (Nonnotte *et al.*, 1993). As the reported ultrastructural damage (Nonnotte *et al.*, 1993) appears to be very similar to that described in this study, a drop in arterial haemolymph partial oxygen pressure may well have provoked the described changes in scaphognathite activity. Instead of resulting in a simple increase in the rate of beating, responses are for more subtle and aimed mainly at the relationships between the two scaphognathites. Such changes in ventilatory activity have not been detected previously, as data analyses often involve the summing of the two scaphognathites to produce a single value (e.g. Jouve-Duhamel and Truchot, 1985).

Many different methods have been used to analyse scaphognathite activity, particularly to allow for the considerable variability in activity, both within the same gill chamber as well as between left and right branchiostegite of the same animal. Such variability makes direct comparisons of data difficult. For example, Jouve-Duhamel and Truchot (1983, 1985) calculated scaphognathite activity levels as the mean of the two scaphognathites, and used the data from a single scaphognathite during periods of unilateral activity. Although this approach facilitates data handling, the statistic may not reflect accurately the animals' physiology (the assumption is made that the unilateral recordings are proportionally higher than bilateral recordings from a single scaphognathite). In the current experiment, the data from each gill chamber were considered separately. Single consideration allows changes in the relationship between the two scaphognathites to be assessed. This approach has rarely been applied to study the ventilatory response of *C. maenas* to environmental variables; however, it has been used for the crayfish *Procambarus simulans* (Larimer, 1964). McDonald *et al.* (1977) postulated that unilateral scaphognathite activity was the preferred behaviour of *Cancer magister* due to a reduction in metabolic costs compared to even very low-rate bilateral scaphognathite activity. In fish, ventilatory activity has relatively high metabolic costs (Jones and Schwarzfeld, 1974) and data available for *Cancer magister* support the same for invertebrates (Johansen *et al.*, 1970).

Dominance of one scaphognathite raises the question whether this behaviour will have repercussions on the condition of the epithelia in the branchiostegite, as a greater

ventilatory volume passing the gills in the active chamber will be concomitant with larger amounts of water-borne metal. Generally, gills were removed from the right branchiostegite, and epithelial damage did not seem to vary according to the dominant scaphognathite. Such a one-sided effect on the gills may, however, be evident if total tissue burdens of copper or metallothionein concentrations were measured in the gills of the two branchiostegites separately.

Apart from ventilatory activity and transfer mechanisms in the respiratory tissues, gill perfusion and haemocyanin oxygen affinity are other important variables in the exchange of gases between the external medium and the haemolymph, and between the gill surfaces and other tissues. As a consequence of the low oxygen carrying capacity of the haemolymph, *C. maenas* expresses a relatively low ventilation/perfusion ratio (1:4) (Taylor and Taylor, 1992). Whether exposure to copper leads to a change in this ratio was not assessed in the present study, as parallel recordings of cardiac rate were not made. Previous studies have described irregular cardiac activity following exposure to relatively high levels of copper (10 mg Cu L^{-1}) (Depledge, 1984a), with a concomitant disruption of the relationship between ventilation and gill perfusion. In *Crangon crangon*, acute exposure to lethal levels of copper lead to a rise in both ventilatory and cardiac activity (Price and Uglow, 1980). Haemocyanin oxygen affinity is closely related to ventilatory and cardiac activity. Although copper does not affect the oxygen binding capacity of haemolymph proteins directly (Truchot and Boitel, 1992), lactate, a by-product of anaerobic metabolism and, therefore, linked to damaged respiratory tissue, can enhance its binding properties (Truchot, 1980). In contrast, acidification of the haemolymph following exposure of *C. maenas* to copper (Nonnotte *et al.*, 1993), reduces haemocyanin oxygen affinity due to proton-binding as part of the Bohr effect (Jokumsen and Weber, 1982). The changes in scaphognathite activity reported above are, therefore, partly a reflection of the effect of copper on respiration at the biochemical level, as well as being related to the cellular gill changes observed.

The present study highlights the link between copper-induced damage at the cellular level within gill tissue and changes in the scaphognathite behaviour of *C. maenas*. Epithelial disorganisation and dysfunction, therefore, may be causing compensatory physiological adjustments. There was little indication of copper detoxification occurring through metallothionein synthesis, and this is consistent with the copper induced changes measured at the cellular and physiological levels.

In summary, copper-exposed animals spend more time in ventilatory activity, with a more equal relationship between the activity levels of right and left scaphognathites and a significantly lower median beat rate than control crabs. These observations were supported by a significant difference in the frequency distributions of the scaphognathite rate data between control and copper-exposed *C. maenas*. Furthermore, bilateral scaphognathite activity increased at the expense of time spent in both bilateral and unilateral apnoea. These findings are summarised in Figure 4.15. Changes at the physiological level occur simultaneously with overt gill tissue damage (especially of the respiratory gill epithelia). Ultrastructural changes may represent a direct cause for physiological change due to resulting tissue hypoxia. Considering the degree of structural damage to the gills, and the severe haemolymph hypoxia reported by Nonnotte *et al.* (1993) following a similar exposure, it is somewhat surprising that oxygen transport to the gills by a rise in gill ventilation was not increased.

Figure 4.1: a. The Computer-Aided Physiological Monitor (CAPMON), comprising infra-red sensors (Se) (attached to the carapace of a specimen of *Carcinus maenas*), the computer-transducer interface (Ct), and a laptop computer for data display and recording. **b.** Sites of collar (Co) and sensor attachment on the carapace of *Carcinus maenas* for the recording of scaphognathite (Sc) and heart (He) activity.


a
Ct
Se

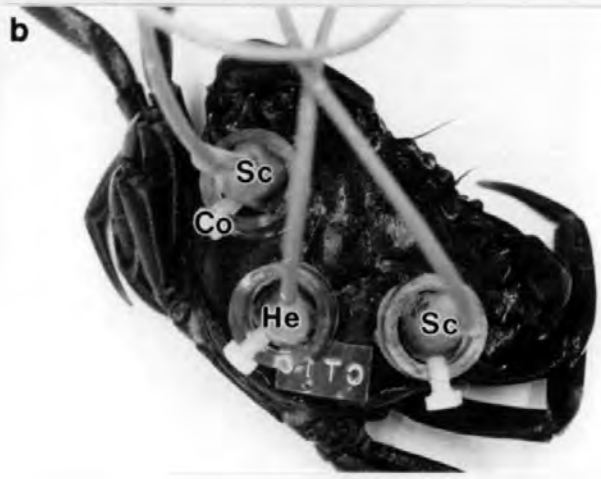
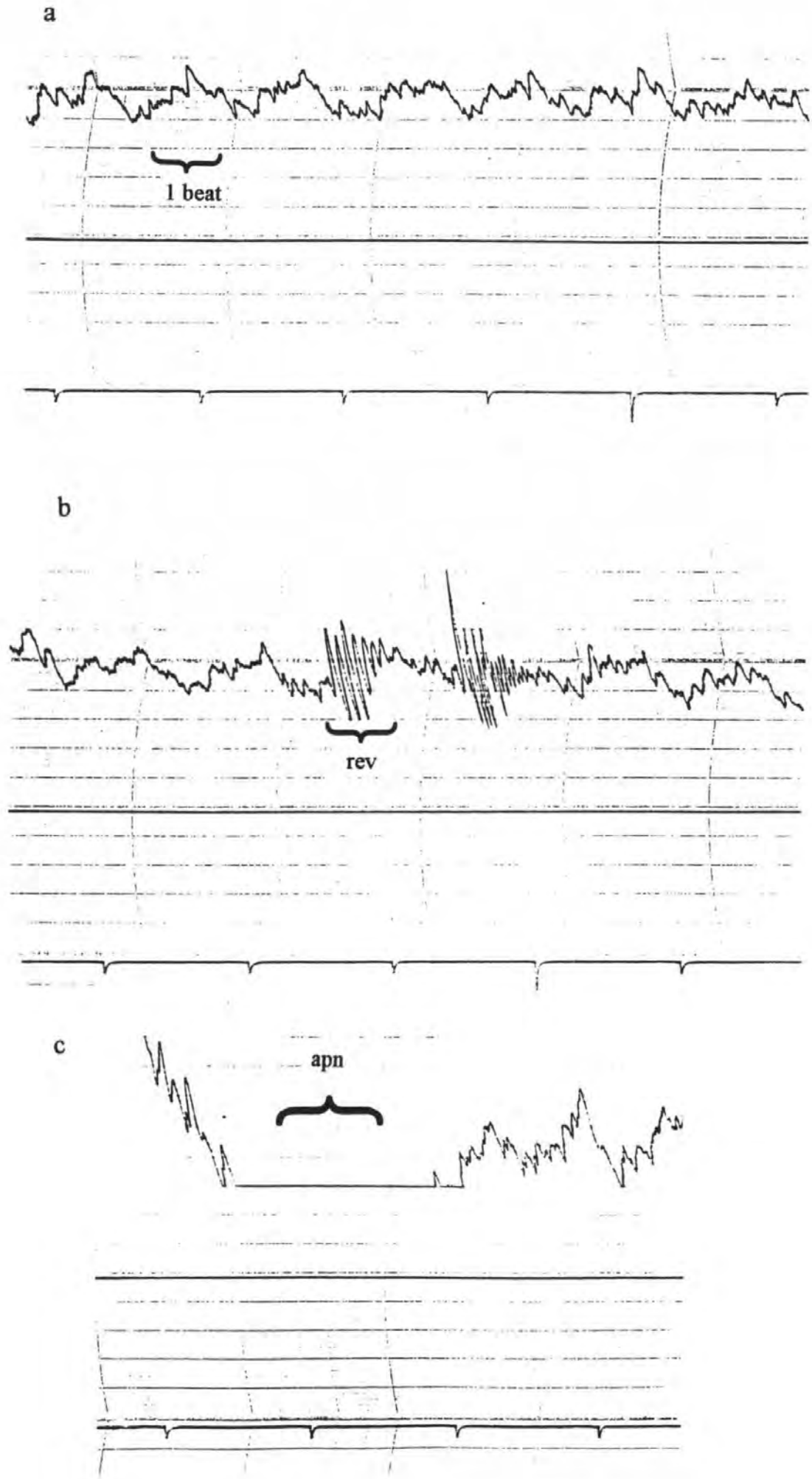

b
Sc
Co
He
Sc

Figure 4.2: Extracts of the trace plotted by the polygraph during monitoring of pressure changes within the gill chamber of *Carcinus maenas* using a pressure transducer. **a.** gradual forward beating of the scaphognathite. **b.** rapid reversed beating (rev). **c.** apnoea period (apn).


a
1 beat
b
rev
c
apn

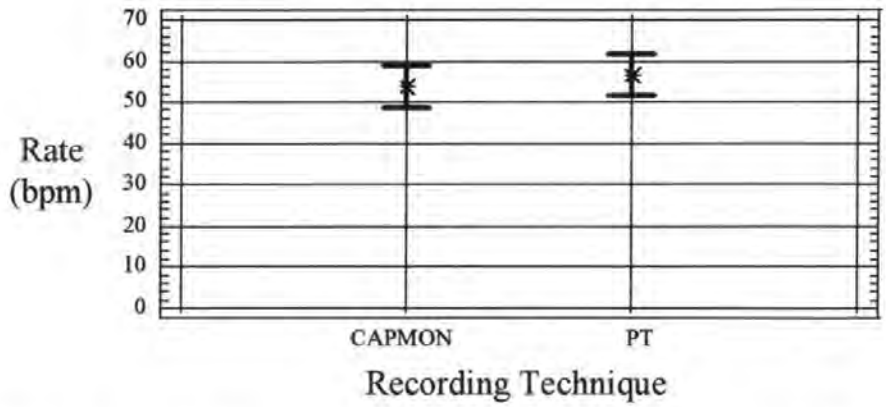


Figure 4.3: Scaphognathite beat rates in beats per minute (bpm) recorded simultaneously in a gill chamber of *Carcinus maenas* by the Computer-Aided Physiological Monitor (CAPMON) and the polygraph attached to the pressure transducer (PT) over a period of 10 min (means and standard errors).

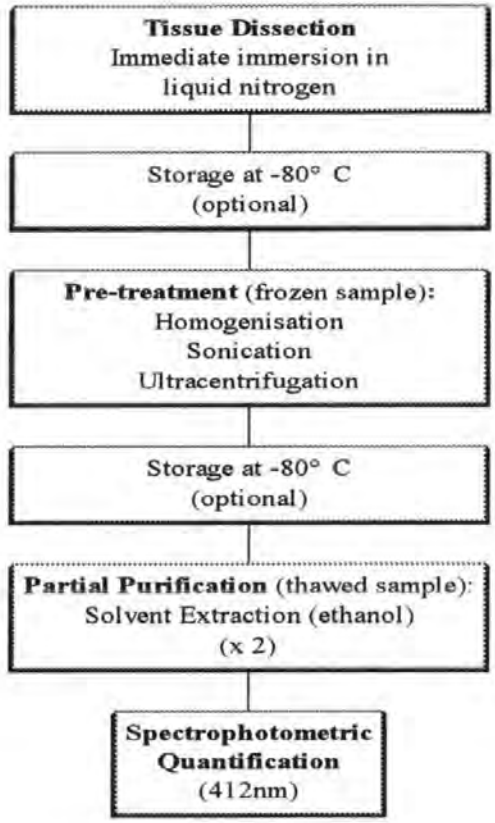


Figure 4.4: Schematic representation of the metallothionein quantification procedure used for tissue of *Carcinus maenas* (method after Pedersen *et al.*, 1994).

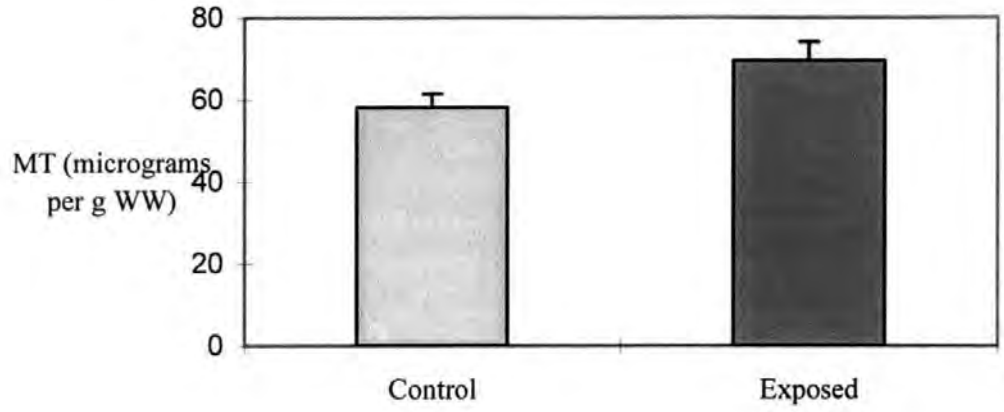


Figure 4.5: Gill tissue metallothionein (MT) concentrations [μg g^{-1} wet weight (WW)] measured in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* (means and standard errors). [n(control)=16; n(exposed)=15]

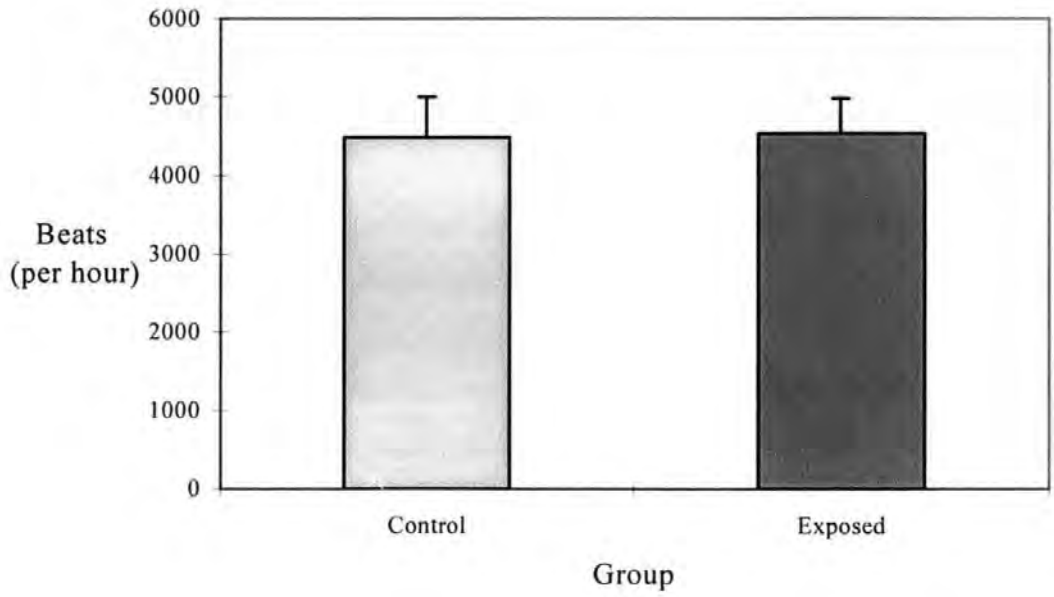


Figure 4.6: Total scaphognathite beats in 1h for control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* (means and standard errors); counts for left and right scaphognathites are added to give one figure per animal. [n=16 for both groups]

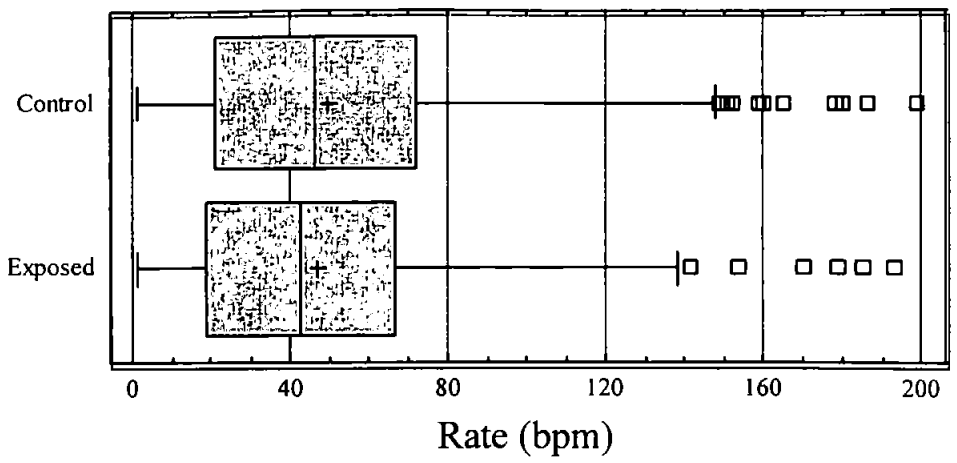


Figure 4.7: Box-Whisker plot* of scaphognathite beat rates in beats per minute (bpm) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas*, during active ventilatory periods, i.e. >0 bpm. [n=16 for both groups]

*see Appendix 3

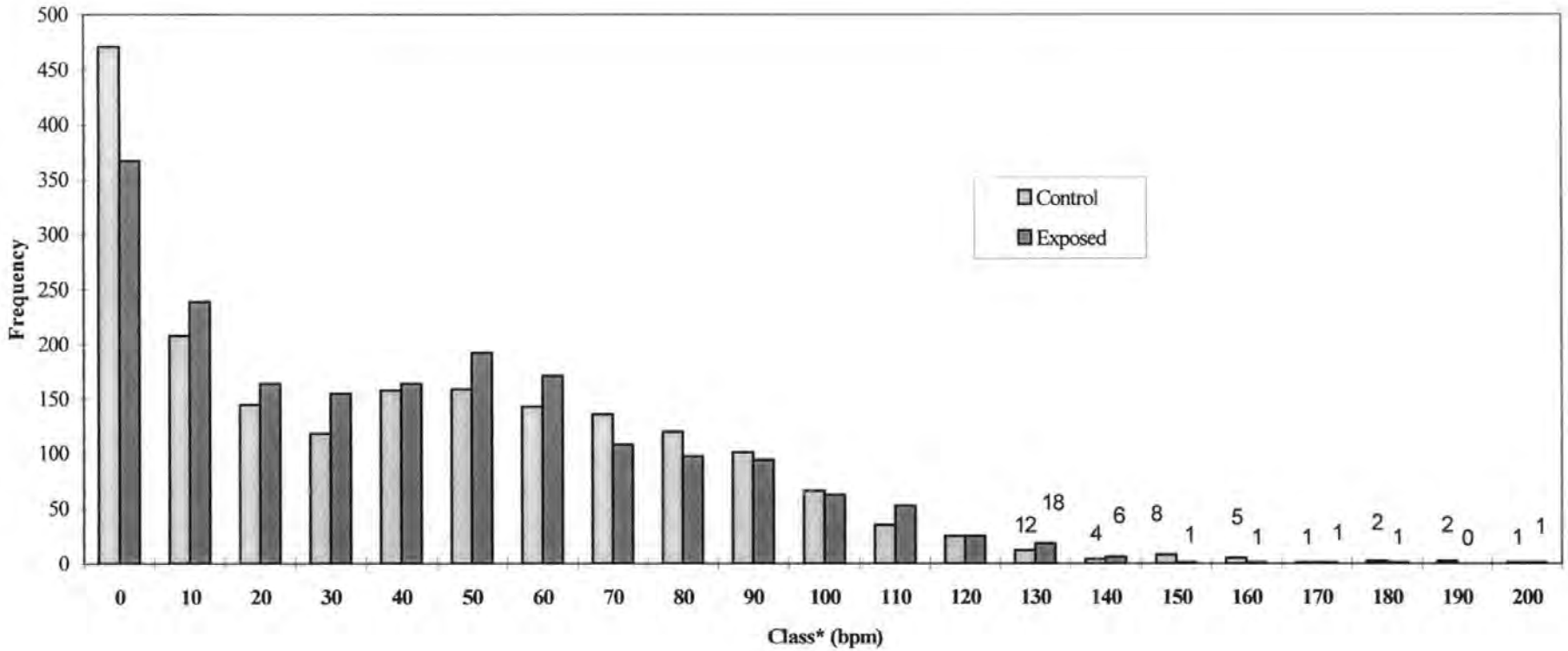


Figure 4.8: Frequency of scaphognathite beat rates in beats per minute (bpm) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas*. Actual data values are given for classes ≥130 beats per minute as frequencies are very low. [n=16 for both groups]

* Class boundaries given are the maximum value included

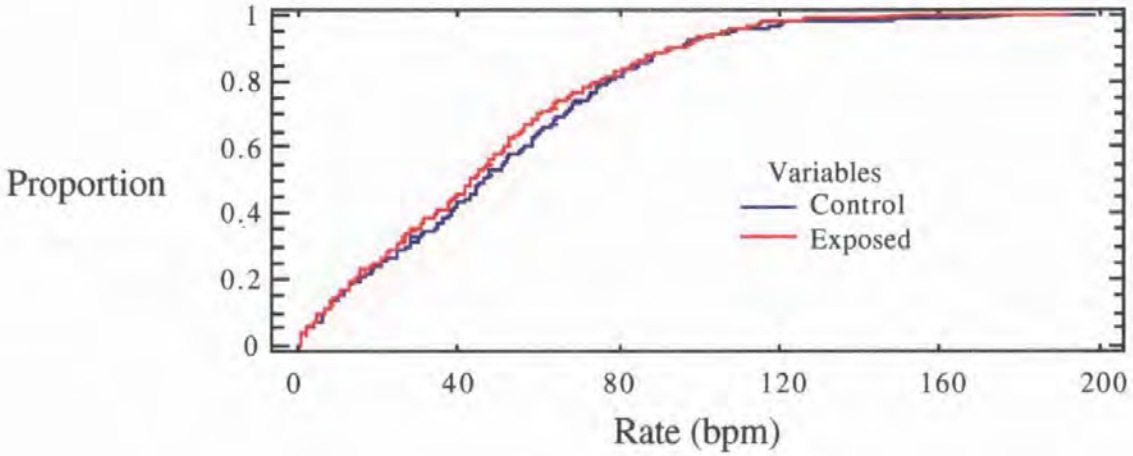


Figure 4.9: Cumulative frequency plot of scaphognathite rate data in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* during active ventilatory periods (>0 bpm). [n=16 for both groups]

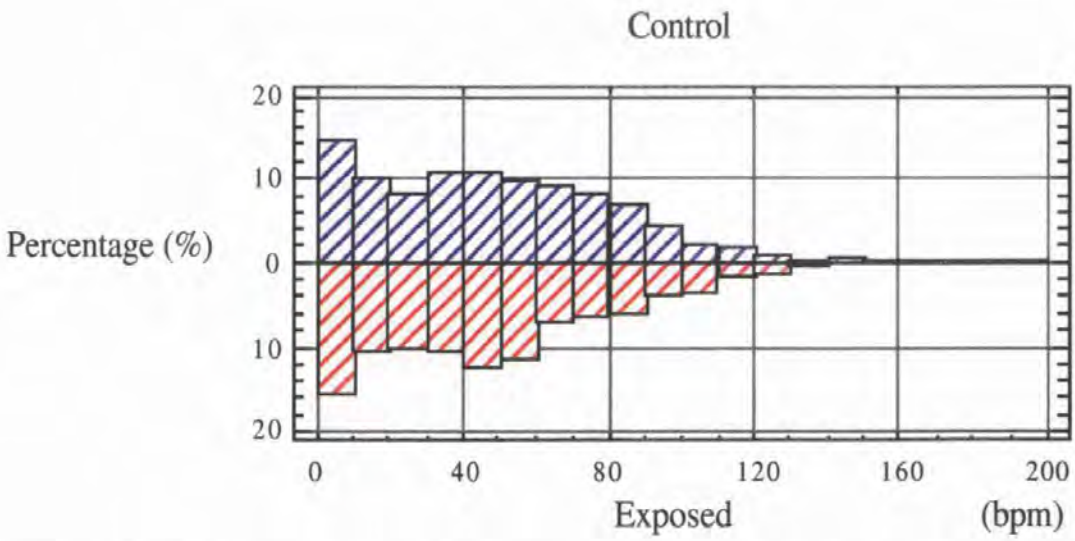


Figure 4.10: Relative frequency histogram of scaphognathite beat rates in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* during active ventilatory periods (>0 bpm). [n=16 for both groups]

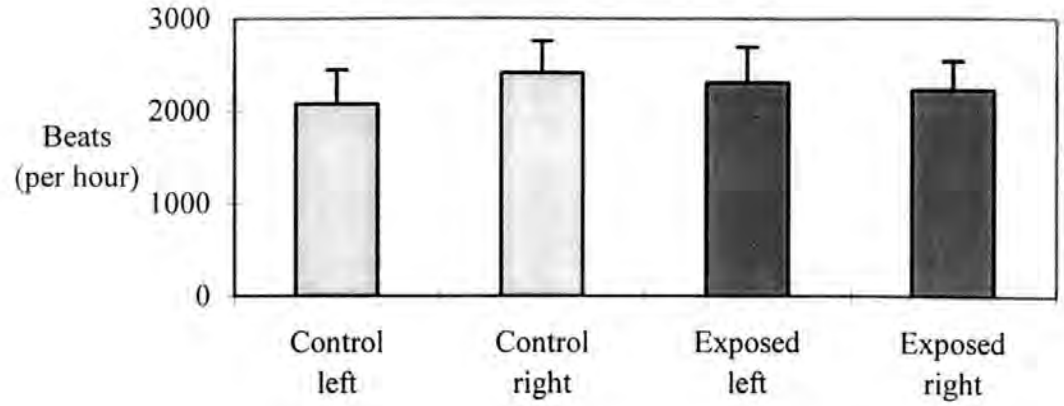


Figure 4.11: Total number of scaphognathite beats in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* recorded over 1 h, distinguishing between left and right scaphognathites (means and standard errors). [n=16 for all groups]

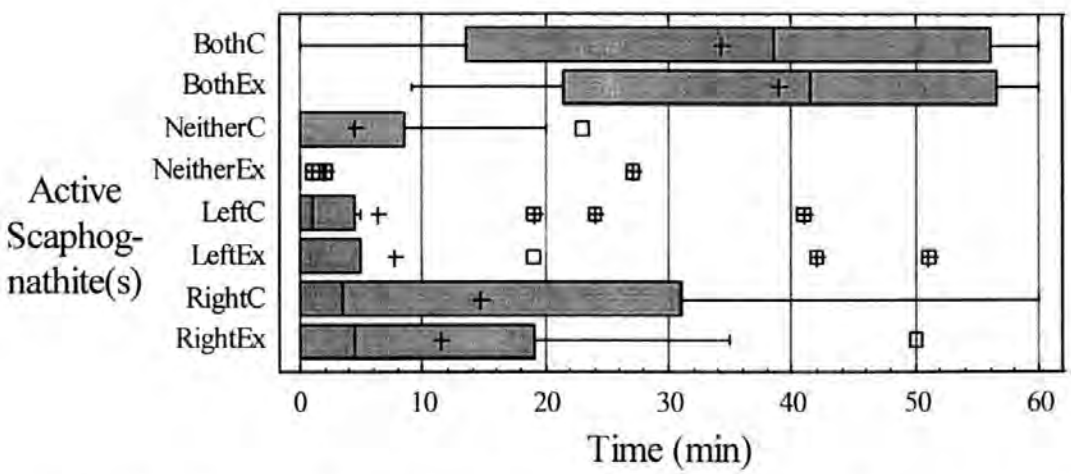


Figure 4.12: Box-Whisker plot* of time spent in bilateral (Both) ventilatory activity, bilateral apnoea (Neither), as well as unilateral (Left/Right) ventilatory activity by control (C) and exposed (Ex) (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* during 1 h. [n=16 for all groups]

*see Appendix 3

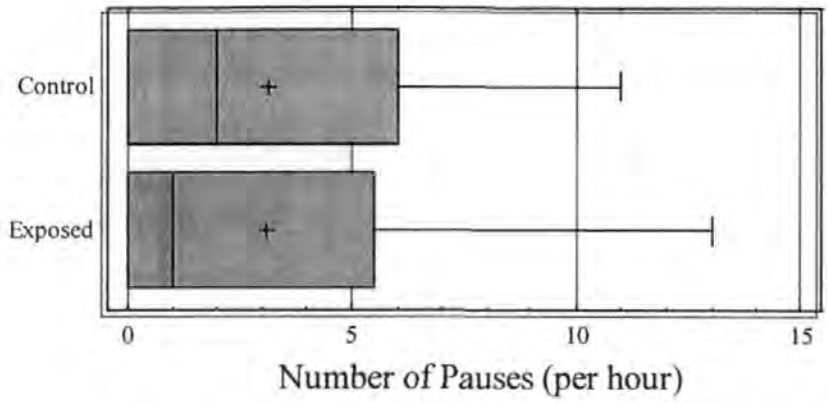


Figure 4.13: Box-Whisker plot* of the number of unilateral ventilatory pauses recorded in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* during 1 h. [n=16 for both groups]

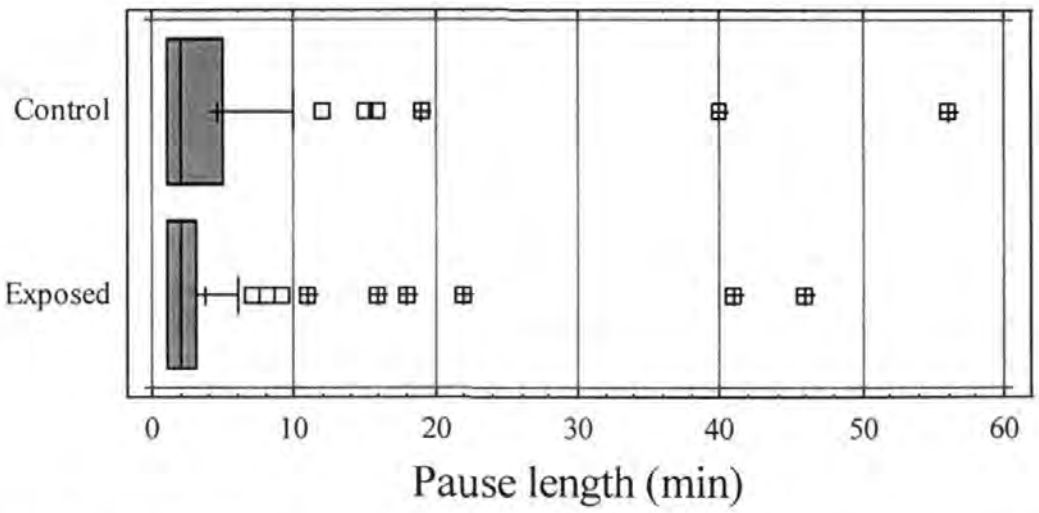


Figure 4.14: Box-Whisker plot* of unilateral ventilatory pause lengths in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* during 1 h. [n=16 for both groups]

*see Appendix 3

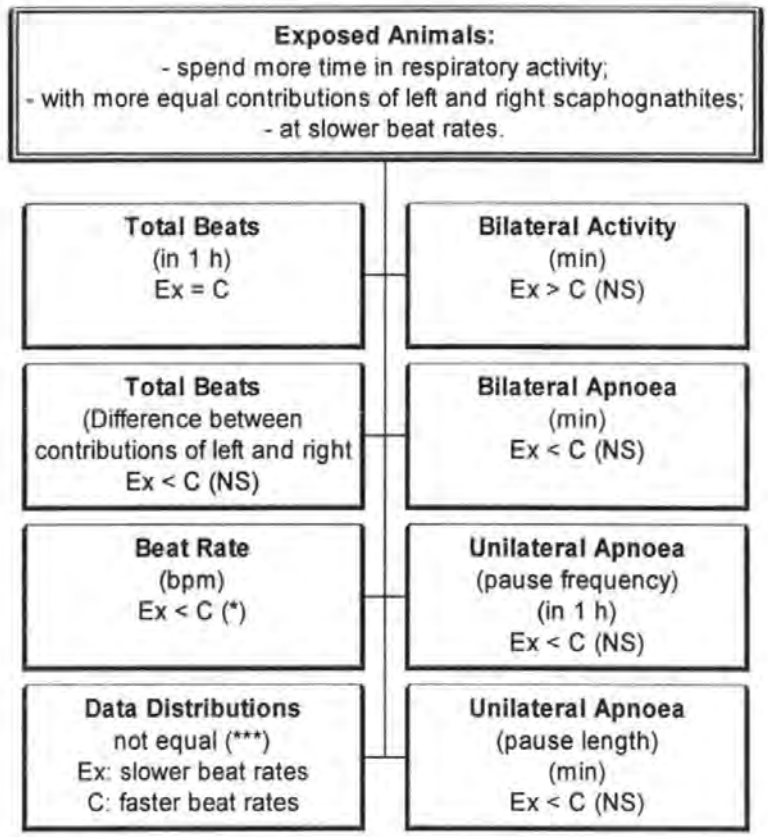


Figure 4.15: Summary of the effects of exposure to copper (10 days at 500 μg Cu L^{-1}) on ventilatory activity in *Carcinus maenas*, leading to an increase in the ventilation volume (theoretical) (C, control animals; NS, not significant; Ex, exposed animals; *, significant [$p<0.05$]; ***, highly significant [$p<0.005$]). [n=16 for control and exposed groups]

Table 4.1: Significance test results of comparisons of median time spent in unilateral (Left/Right) and bilateral (Both) scaphognathite activity, as well as bilateral apnoea (Neither) by control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas*, including p-values and Mann-Whitney test statistics (W). [n=16 for all groups]

Comparison	p-Value	Test Statistic (W)
Both (Control) ≠ Both (Exposed)	0.5459	144.5
Neither (Control) ≠ Neither (Exposed)	0.2348	102.5
Left (Control) ≠ Right (Control)	0.3495	152.0
Left (Exposed) ≠ Right (Exposed)	0.2973	154.0

CHAPTER 5

EFFECTS OF COPPER AND TEMPERATURE ON SCAPHOGNATHITE ACTIVITY IN *CARCINUS MAENAS*, WITH REFERENCE TO GILL ULTRASTRUCTURE AND METALLOTHIONEIN

Abstract

Exposure of *Carcinus maenas* to 500µg Cu L^{-1} for 10 days resulted in severe damage of the respiratory epithelia and, to a lesser degree, the osmoregulatory epithelia. These ultrastructural changes occurred despite a significant increase in the concentration of metallothionein within the gill tissue, indicating that the copper detoxification process had been activated. Tissue damage and detoxification appeared simultaneously with changes in scaphognathite activity. At 15°C and at 21°C, copper-exposed animals displayed lower overall scaphognathite beat rates than control animals, although the total number of scaphognathite beats decreased at the lower and increased at the higher temperature. The time spent in bilateral ventilatory activity was less in copper-exposed animals at 15°C, but more at 21°C, compared with control animals at the same temperatures. The proportion of time spent in bilateral and unilateral apnoea increased with copper exposure at 15°C but decreased at 21°C.

With the rise in temperature from 15°C to 21°C, control animals spent more time in bilateral scaphognathite activity, as well as bilateral and unilateral apnoea, resulting in a more equal contribution by both scaphognathites to the total number of scaphognathite beats than in copper-exposed individuals. In contrast, copper-exposed animals responded to the increase in temperature by a reduction in the time spent in bilateral activity, as well as unilateral and bilateral apnoea.

The observed changes in scaphognathite activity may represent a means of altering ventilation volumes, and thus oxygen transport to the gills. These adjustments may be occurring in response to metabolic changes incurred by the increase in temperature, as well as compensatory reactions to copper-related tissue damage and, therefore, impaired transfer of gases.

5.1 Introduction

Crustaceans inhabiting coastal and estuarine areas are subject to a range of anthropogenic contaminants derived from urban and industrial developments, including significant levels of trace metals from atmospheric inputs, river discharge, industrial waste and sewage sludge (Clark, 1992). Although much is known about the effects of trace metals on aquatic animals from laboratory studies, especially in terms of their respiration (see review by Spicer and Weber, 1991), these investigations frequently do not reflect the consequences of exposure to the contaminant in the field, where the animals face fluctuations in salinity, oxygen levels and temperature. For example, *Carcinus maenas*, inhabiting relatively shallow coastal and estuarine areas, often remains stranded in rock pools at low tide and is exposed subsequently to temporary changes in environmental temperature, as well as contaminants in the water (Naylor, 1962). The significance of exposure to trace metals (and other contaminants) for the animal's physiological and reproductive fitness, therefore, depends on stress factor synergies (Depledge, 1987). Such interactions may involve an increase in toxicity of the metal due to speciation or exacerbate contaminant effects physiologically (e.g. by making the animals cope with an increase in environmental temperature as well as metal-induced gill damage) (Lawson *et al.*, 1995; Hebel *et al.*, 1997).

It is widely recognised that temperature influences the toxicity of trace metals to aquatic crustaceans (McLeese, 1974; Chassard-Bouchaud, 1980; Denton and Burdon-Jones, 1981; Gaudy, 1983; McLusky *et al.*, 1986; Rengel de Zambrano *et al.*, 1993). There is, however, relatively little information available on the combined effects of temperature and trace metal exposure on the respiratory behaviour in Crustacea (Cairns *et al.*, 1975; Gaudy, 1983), particularly ventilatory activity (but see Cumberlidge and Uglow, 1977b; Price and Uglow, 1980; Spicer and Weber, 1992). Many factors, such as environmental hypoxia (e.g. Taylor, 1976), salinity (e.g. Taylor, 1977) locomotory activity (e.g. McMahon *et al.*, 1979) and gill parasite infestation (Gannon and Wheatly, 1992) are known to affect ventilatory rate in crabs. Previously, an increase in temperature has been shown to cause changes in ventilatory activity of *C. maenas*, including an increase in scaphognathite beat rate (Cumberlidge and Uglow, 1977b), and changes in the frequency and duration of scaphognathite reversals (Taylor and Wheatly, 1979). The current study assesses the effect of temperature increase on the scaphognathite activity of copper-exposed *C. maenas*, linking changes of physiology with structural gill tissue damage and concentrations of metallothionein.

5.2 Materials and Methods

5.2.1 Collections and holding conditions

In May 1996, 32 intermoult male *Carcinus maenas* with green carapace and of approximately equal size (carapace width *c.* 6 cm) and weight (50-60 g wet weight) were collected from the Avon Estuary, near Bantham (Devon), using drop nets baited with fish (various species). Transport and aquarium holding conditions were as described in Section 3.2.3. Two exposure and two control tanks (each containing 4 animals), were moved from the 15°C to 24°C aquarium 18 h prior to the start of physiological monitoring to allow the water to reach a higher final experimental temperature of 21°C.

5.2.2 CAPMON experimental procedure

Following 9 days in the experimental tanks at 15°C, by which time any tidal rhythmicity in behaviour had been lost (Arudpragasam and Naylor, 1964b), crabs were fitted with CAPMON collars (see Section 3.2.1). After a further 24 h, the infra-red sensors were fitted (i.e. after 10 days in the experimental tanks), and the crabs were moved into aerated 2 L plastic tanks, covered with black polythene to exclude visual stimuli to the crabs. These smaller tanks contained the same experimental media as the glass experimental tanks (clean seawater or seawater with 500 μg Cu L^{-1} at 15° or 21°C). The crabs remained undisturbed for 1 h prior to physiological monitoring (to allow return to resting-level scaphognathite activity). For each crab, the activity of both the right and left scaphognathite was recorded over a period of 60 min using CAPMON (see Section 3.2.1). In the control and exposed groups, scaphognathite activity was monitored at 15° or 21°C at the same time on consecutive days. The CAPMON equipment (sensors and transducer/computer interfaces) was randomised between groups. The data were analysed using parametric statistical tests as far as possible, however, in many cases non-parametric tests were necessary due to the non-normal distribution of data.

In the control and copper-exposed animals at each experimental temperature, scaphognathite activity was assessed as the total number of scaphognathite beats during 1 h (Multifactor ANOVA, 2-way interaction). The median scaphognathite beat rates (per minute) of control and copper-exposed animals at the two temperatures (using all data >0 bpm) were compared using Mann-Whitney Two-sample Comparisons. The distribution of the data was analysed using Kolmogorov-Smirnov Two-sample Tests, to test for differences in preferred beat rates of the animals. The relationship between left and right scaphognathites was assessed by establishing the relative contributions made by each of the left and right scaphognathites to the total beats per hour and the amount of time spent

in bilateral ventilatory activity (Multifactor ANOVA, 2-way Interaction; Mann-Whitney Two-sample Tests). The amount of time spent in bilateral apnoea (i.e. neither scaphognathite active) by the different groups was assessed using the Kruskal-Wallis Multiple-sample Comparison. Unilateral activity (i.e. either left or right scaphognathite active) and the number and duration of periods of unilateral apnoea were compared using Kruskal-Wallis Multiple-sample Comparisons and Mann-Whitney Two-sample Comparisons. All statistical tests were carried out using *Statgraphics Plus for Windows (Version 2.1)*.

5.2.3 Ultrastructural gill analysis

Following physiological monitoring, a posterior (number 8) and an anterior gill (number 5) was removed from the right gill chamber of all control and copper-exposed crabs. Tissue processing for electron microscopical analysis was carried out as described in Section 2.2. Analysis of gill tissue was carried out both qualitatively and quantitatively. The latter involved determination of the number of haemocytes and nephrocytes present in a 300 μm lamellar section, choosing the tissue portion distal to the marginal canal (see Section 1.3.1). Comparisons were made between control and copper-exposed gill tissues only (i.e. including animals monitored at 15°C and 21°C) to achieve sufficiently large samples to allow statistical analysis.

5.2.4 Metallothionein quantification

Gill tissue, not used for electron microscopical study, was removed from the gill chambers and snap frozen in liquid nitrogen. Quantification of metallothionein in the tissue was carried out as described in Section 3.2.6. Data were analysed by One-way ANOVA using *Statgraphics Plus for Windows (Version 2.1)*.

5.3 Results

5.3.1 Gill ultrastructure

Following exposure to 500 μg Cu L^{-1} for 10 days, the ultrastructure of the anterior and posterior gills was identical to that described in Section 2.3.5 and is, therefore, not repeated in detail here. The brief exposure of crabs to the higher temperature did not affect gill ultrastructure. In summary, epithelial damage included cellular necrosis, vacuolation and hyperplasia, and infiltration of epithelia by haemocytes. Initiation of the detoxification process, as indicated by the presence of lipofuscin granules, was seen in the posterior gill tissue. In general, the anterior respiratory gills, and respiratory epithelia within the

osmoregulatory gills, showed the highest level of damage resulting from exposure to copper. The ultrastructural changes observed are summarised in Figures 2.10 and 2.11.

Anterior gills of control animals contained significantly fewer haemocytes than anterior gills of copper-exposed animals (Mann-Whitney Two-sample Test; W=169.0; p=0.000007) (Fig. 5.1). There was no significant difference between the number of haemocytes in posterior gills of control and copper-exposed crabs (Mann-Whitney Two-sample Test; W=113.0; p=0.4987) (Fig. 5.1). Haemocyte numbers in the anterior and posterior gills of control animals were not significantly different (W=85.5; p=0.8062), however, in the copper-exposed animals, there was a higher median number of haemocytes in the anterior than the posterior gills (W=19.0; p=0.0003) (Mann-Whitney Two-sample Test) (Fig. 5.1).

The number of nephrocytes in the anterior gills of control animals was not significantly different to counts in anterior gills of exposed animals (Mann-Whitney Two-sample Test; W=88.0; p=0.8688) (Fig. 5.2). There were, however, significantly more nephrocytes in the posterior gills of control than the exposed animals (Mann-Whitney Two-sample Test; W=27.5; p=0.0006) (Fig. 5.2). Comparisons of anterior and posterior gills within the same experimental group of crabs showed a lower median number of nephrocytes in the anterior than the posterior gill of the control animals (W=169.5; p=0.00006), but no significant difference between anterior and posterior gills of the exposed animals (W=113.0; p=0.2776) (Mann-Whitney Two-sample Test) (Fig. 5.2).

5.3.2 Metallothionein

Metallothionein concentrations in the gill tissue of *C. maenas* were significantly elevated following exposure to 500 μg Cu L^{-1} for 10 days compared with control concentrations (Kruskal-Wallis Two-sample Test; test statistic=4.82; p=0.0281) (Fig. 5.3).

5.3.3 Scaphognathite activity

Analysis by Multifactor ANOVA (2-way Interaction) showed that neither temperature (F=0.2; p=0.6621) nor exposure to copper (F=0.16; p=0.6917) affected the total number of scaphognathite beats recorded for each group (Fig. 5.4). There was no significant interaction between the two factors (F=1.48; p=0.2353).

Comparison of beat rates, excluding periods of apnoea, using Mann-Whitney Two-sample Comparisons, showed that the median beat rate (per minute) was significantly lower in exposed than control animals (at 15° and 21°C) and significantly higher in control animals at 21°C than at 15°C (Fig. 5.5; Table 5.1). There was, however, no significant

difference in scaphognathite beat rate between exposed animals at the two temperatures (Table 5.1).

The frequency distributions of scaphognathite beat rate (bpm) data (including periods of apnoea) are illustrated in Figures 5.6 a and b. At 15°C, control animals spent relatively less time in ventilatory rest (0 bpm) than exposed animals at the same temperature. This relationship was reversed at 21°C, where the scaphognathites of control animals spent more time inactive. Analysis (excluding periods of apnoea) using Kolmogorov-Smirnov Two-sample Tests showed significant differences in distributions (Table 5.2). At 15°C, control crabs spent a greater proportion of ventilatory active time at beat rates >60 bpm (the dominant rate lying between 61 and 70 bpm) than exposed animals at the same temperature. The latter spent a greater proportion of time at rates <60 bpm (the dominant rate lying between 1 and 10 bpm) (Figs 5.7 and 5.8). At 21°C, control crabs spent more time at beat rates exceeding 100 bpm than exposed animals, and the data distribution was more even than in exposed animals, no dominant beat rate being discernible (Figs 5.9 and 5.10). In exposed animals, however, the dominance of beat rates between 1 and 10 bpm is more pronounced. The overall highest beat rate is displayed by exposed animals at 21°C, although this beat rate (360 bpm) has a very low frequency. At 15°C, control animals spent a smaller proportion of time at low beat frequencies (<60 bpm) than control animals at 21°C (Fig. 5.11). At the lower temperature, a distinct preference for beat rates between 50 and 80 bpm is visible, whereas this preference is lost at the higher temperature, with the frequency of beat rates being more equally distributed throughout the range (Fig 5.12). For exposed animals, the distribution of data was similar at both temperatures (Fig. 5.13), with a dominance in beat rates between 1 and 10 bpm (Fig. 5.14). At 15°C, there was a second peak in the frequency distribution at beat rates between 61 and 70 bpm. In both control and exposed animals, the highest beat rates were displayed by animals at 21°C rather than 15°C.

Figure 5.15 illustrates the contributions made to the total number of scaphognathite beats per group by left and right scaphognathites. The values for left and right scaphognathites were not significantly affected by either copper or temperature (Multifactor ANOVA, 2-way Interaction) (Table 5.3). Differences in the relative activity of the two scaphognathites, however, were greater at 15°C, a more equal relationship being present at 21°C (Table 5.3). Interactions between the two factors copper and temperature were not significant in either case (Table 5.4).

The time animals spent with both scaphognathites active simultaneously was not significantly affected by exposure to copper (F=3.38; p=0.0782), or influenced by the increase in temperature (F=0.05; p=0.8247) (Multifactor ANOVA, 2-way Interaction) (Fig. 5.16). The interaction between the two factors (copper and temperature), however, was significant (Multifactor ANOVA, 2-way Interaction; F=9.87; p=0.0044); the rise in temperature in control animals leading to a decrease in the time spent in bilateral activity, but leading to an increase in copper-exposed animals. Further comparisons were, therefore, made using Mann-Whitney Two-sample Tests which showed that control animals at 15°C spent significantly more time in bilateral activity than exposed animals at the same temperature and at the higher temperature (Table 5.5). At 21°C, control animals spent significantly more time with both scaphognathites active than exposed animals at 15°C. The latter spent significantly less time in bilateral ventilatory activity than the exposed animals at the higher temperature (Table 5.5).

The time spent with neither scaphognathite active (bilateral apnoea) by control and exposed animals at the different temperatures did not differ significantly (Kruskal-Wallis Multiple-sample Comparison; test statistic=1.08; p=0.7827) (Fig. 5.17). Although the medians of the four groups are the same (=0), the shape of the Box-Whisker plot (Fig. 5.17) does indicate some differences in the data; control animals at 15°C spending less time in bilateral apnoea than exposed animals at the same temperature. At 21°C, the opposite is evident, control animals spending more time in bilateral apnoea than exposed animals. In control animals, therefore, increase in temperature lead to an increase in the time spent in bilateral apnoea. In exposed animals, there was a reduction in the time spent in bilateral apnoea at the higher temperature.

The time spent in unilateral ventilatory activity by either right or left scaphognathites did not differ significantly between control and exposed animals at the two temperatures (Kruskal-Wallis Multiple-sample Comparison; F=7.24; p=0.4045) (Fig. 5.18).

Comparison of pause lengths in the four groups showed significant differences in the median duration of pauses (Kruskal-Wallis Multiple-sample Comparison; test statistic=21.75; p=0.00007) (Fig. 5.19). Mann-Whitney Two-sample Comparisons showed that the duration of pauses was significantly shorter in control animals at 15°C than 21°C, however, the opposite was true in exposed animals (the increase in temperature resulted in a significant decrease in pause length) (Table 5.6). Unilateral ventilatory pauses were

significantly shorter in control animals at 15°C than in exposed animals at the same temperature, and significantly longer at 21°C (Table 5.6).

The number of unilateral pauses occurring in animals of all groups was not significantly different (Kruskal-Wallis Multiple-sample Comparison; test statistic=2.53; p=0.4694) (Fig. 5.20).

In summary, the increase in temperature from 15°C to 21°C caused a significant rise in the scaphognathite beat rate of control animals, significant differences in data frequency distributions in control and copper-exposed animals, a significant increase (control crabs) or decrease (copper-exposed crabs) in the time spent in bilateral activity, and a significant decrease (control crabs) or increase (copper-exposed crabs) in the length of unilateral apnoea periods.

Exposure to copper caused a significant increase in scaphognathite beat rate at 15°C and 21°C, significant differences in the frequency distributions of data, a significant decrease in the time spent in bilateral ventilatory activity of animals at 15°C and a significant increase (15°C) or decrease (21°C) in the duration of unilateral apnoea periods.

Statistically significant results, as well as observed trends, are summarised in Tables 5.7 and 5.8.

5.4 Discussion

Exposure of *C. maenas* to 500 μg Cu L^{-1} for 10 days resulted in the same ultrastructural gill degeneration as described in Section 2.3.5, following a 14 day exposure to the same copper concentration.

Morphometric analysis showed that exposure to copper leads to an increase in the number of haemocytes in the anterior gills, especially integrated into the hyperplasic multilayered epithelia. Such an increase was not observed in the posterior osmoregulatory gills, implying that the respiratory epithelia are more sensitive to copper exposure than the osmo-/ionoregulatory epithelia (in terms of epithelial cell degeneration and changes in the frequency of haemocytes). The role of the increased numbers of haemocytes present in the gill tissue is uncertain, especially since recent findings by Rtal *et al.* (1996) illustrated that these cells are not involved directly in the detoxification of exogenous copper, as previously suggested (Lawson *et al.*, 1995) for *C. maenas*. Increased numbers of haemocytes in the gill tissue, however, are concomitant with the presence of more lysosomes, which play an important role in the sequestration of metals (Viarengo *et al.*,

1981). Indeed, in *Scylla serrata*, haemocytes were found to play a significant role in the detoxification of exogenous copper (Balaji *et al.*, 1989). Since haemocytes are structurally degenerate [including damage to the cell membrane, leading to the distribution of haemocyte organelles (nuclei, granules, lysosomes) within the surrounding epithelial tissues], any functional purpose attributed to their increased numbers seems doubtful. They may, however, be present to meet an increase in energy demand (caused by the heightened cell turnover) through carbohydrate metabolism (Johnston *et al.*, 1973). Damage to lysosomal membranes, caused by excessive amounts of copper in the cytosol, may be partially responsible for the severe cell damage, as this will lead to the leakage of lytic enzymes into the surrounding tissue (Sternlieb and Goldfischer, 1976). Epithelial hyperplasia (leading to partial blockages of the haemolymph space) may be acting as a barrier to the passage of haemocytes. Their presence may, therefore, merely have a mechanical rather than physiological cause, i.e. the thickened epithelia forming a barrier to the passage of haemocytes.

Nephrocytes are involved in endocytotic processes within the gills, removing cell debris (Smith and Ratcliffe, 1981), and fulfil an important function in keeping haemolymph channels unblocked and respiration physically unimpaired. Nephrocytes aid the "cleansing" of the crustacean haemolymph by detoxification as well as filtration and regulation of haemolymph components (Doughtie and Rao, 1981). In control animals, the posterior gills contained significantly more nephrocytes than anterior gills. One reason for this biased distribution of nephrocytes may be related to the difference in lamellar size between the anterior and posterior gills in relation to the size of nephrocytes. Accumulations of nephrocytes, which can reach up to 50μm in diameter (Taylor and Taylor, 1992), in the haemolymph space would undoubtedly lead to blockages of the haemolymph channel. In the anterior gill lamellae, the haemolymph channel may be only 15 μm wide, despite the relatively thin epithelial layer characteristic of the respiratory gill. Such blockages would be detrimental to the physiological functioning of the gill by impairment of haemolymph flow. Lawson (1994) reported a similar distribution of nephrocytes within the posterior gill of *C. maenas*. Nephrocytes were reported to be rare in the distal gill lamellae (which are relatively small), but abundant in the large mid and proximal gill lamellae (Lawson, 1994). It is, therefore, likely that the distribution of nephrocytes is dependent upon their requirement for space. The prevalence of nephrocytes in the proximal gill regions of the posterior gill may be due to their role as the first line of defence against toxic substances, their location coinciding with the site of greatest

ventilatory currents (Lawson, 1994), as well as the entry site of haemolymph to the gill as a whole via the infrabranchial sinuses (Taylor and Taylor, 1992). The biased distribution of nephrocytes may, however, reflect functional differences between gill types and lamellae. Lawson (1994) proposed a predominantly respiratory function for the distal lamellae within the posterior gill (due to the dominance of chief cells in the epithelial layer). Since respiratory function, and small lamellar size, generally occur together, and nephrocytes are not implicated as participants in gaseous exchanges, the size restriction is the most likely cause of nephrocyte distribution. This difference in nephrocyte numbers according to gill type is lost in the gills of copper-exposed crabs, due to a significant reduction in numbers in the posterior gills of exposed animals compared to the same gill type in control animals. This observation is surprising, considering the involvement of nephrocytes in endocytotic processes; an increase in cellular material requiring break-down following tissue copper damage should be met by a larger (rather than smaller) number of nephrocytes. The relative decrease in the number of endocytotic cells present may have mechanical reasons, as the thickening of gill epithelia, and thus narrowing of the haemolymph space, may hinder the passage of large nephrocytes into the gills.

The concentrations of metallothionein in the gills increased significantly following 10 days exposure to 500 μg Cu L^{-1}, indicating that influx of exogenous copper leads to an increase in synthesis of these metal-binding proteins. This detoxification mechanism, however, was not successful in preventing severe ultrastructural tissue damage caused by the toxicity of the exogenous copper within the cytoplasm (Sternlieb and Goldfischer, 1976).

These results, together with earlier studies of the haemolymph physiology in *C. maenas* following exposure to copper (Nonnotte *et al.*, 1993), reflect a degree of functional impairment in the gills, which is likely to result in physiological compensatory reactions in the animals, to guarantee the supply of oxygen to the gills. The total number of scaphognathite beats (in 1 h) [assumed to be equivalent to the total ventilation volume passing through the gill chambers (Cumberlidge and Uglow, 1977b)] was not significantly different between control and copper-exposed crabs. The increase in temperature, however, did appear to result in a more equal relationship in the total number of beats recorded from the left and right scaphognathites in both control and exposed animals. This may be caused by the inadequacy of unilateral ventilatory activity in meeting the rise in the metabolic oxygen demands of tissues as a result of the increase in temperature. This trend, however, was not statistically significant.

In exposed animals, scaphognathite beat rate was significantly lower than control values at each temperature. This observation is supported by numerous oxygen consumption measurements in Crustacea [see review by Spicer and Weber, 1991]. Although scaphognathite activity in these studies was not monitored directly, it was suggested that the observed reductions in oxygen consumption following exposure to trace metals were due to changes in ventilatory activity (Spicer and Weber, 1991). Such a direct relationship between oxygen consumption and scaphognathite rate has been reported for squat lobsters, (*Munida rugosa* and *M. sarsi*), where oxygen consumption and scaphognathite rate were measured simultaneously (Zainal *et al.*, 1992). In contrast with the present study, Price and Uglow (1980) reported an increased scaphognathite rate in *Crangon crangon* following exposure to 0.1 to 20 mg Cu L^{-1}. This difference may be explained by the dependence of the response on the exposure concentration of trace metal, which, in the experiments with *C. crangon*, was close to the lethal level (Price and Uglow, 1980).

Increased ventilatory rate with increasing temperature, as shown here in control animals, has been reported previously in *C. maenas* (Cumberlidge and Uglow, 1977a). Direct comparison with previous work, however, is not possible as Cumberlidge and Uglow (1977a) used crabs which were beating at "elevated levels" as opposed to the resting scaphognathite beat activity reported here. In addition, Cumberlidge and Uglow (1977a) give no information as to how animals were exposed to the different water temperatures. Reduction in ventilatory rate at the upper temperature limits has been reported for other Crustacea and interpreted as an inability to sustain the higher metabolic rate caused by the rise in temperature (Varo *et al.* 1991). For example, exposure of the squat lobsters *M. rugosa* and *M. sarsi* to temperatures outside of their normal range lead to a decrease in scaphognathite activity (Zainal *et al.*, 1992). The temperatures chosen for scaphognathite measurements in the present study were within the range encountered by *C. maenas* and any ventilatory changes in response to the increase in temperature would occur with the animals remaining submerged. The threshold for "emigration" in crabs acclimated to 15°C was 28°C (Taylor and Wheatly, 1979). Physiological impairment, due to copper-induced tissue damage, may reduce this temperature threshold, leading to this response. The lower ventilatory rate calculated in the present study, however, is likely to be a statistical artefact. Although the medians of the scaphognathite beat data do indicate a fall in ventilatory rate of the copper-exposed animals at 21°C, mean rate values indicate a rise in ventilatory rate in exposed as well as control animals. A comparison of means,

however, was not possible due to the distribution of the data (even following attempts of data transformation) (Fig. 5.5). The highest beat rates (per minute) reached by the experimental animals were recorded at 21°C, both in control and exposed animals (Fig. 5.6b), which supports the hypothesis that an increase in temperature leads to a simultaneous increase in beat rate of copper-exposed animals.

The range of ventilatory rate data is greater for copper-exposed animals at 21°C than for any of the other experimental groups, indicating that simultaneous exposure to copper and higher temperature increases the variability in the response of *C. maenas*. Such an increase in the variability of physiological measurements has been suggested as evidence for stress effects on organisms (e.g. due to contaminants or changes in environmental variables) (Depledge, 1990a; Depledge and Lundebye, 1996).

Data frequency distributions of measurements from control crabs show that, at 15°C, there is a marked dominance of beat rates in the range 50-80 bpm, a characteristic also present in exposed animals at that temperature. The changes in ventilatory rate with increasing temperature are, however, not associated with a shift of this peak in frequency distributions, but with the disappearance of any peak and a more even distribution of the frequency of observed beat rates. As mentioned above, the "stress" imposed on the animals, in this case by the increase in temperature, leads to a greater variability in response. At both temperatures, exposed animals spend a high proportion of time at very low beat rates of 1 to 10 bpm. This indicates that exposed animals spend more time in unilateral apnoea than indicated by the per minute-recordings made by CAPMON (see below), as rate measurements of 1-10 beats per minute imply that part of that minute was spent in ventilatory rest.

At 15°C, differences in the total beats (per hour), which corresponds to the total ventilation volume (Cumberlidge and Uglow, 1977b), compliment changes in ventilatory rate (i.e. a reduction in the total number of beats in exposed animals compared to control animals at that temperature being concomitant with a decrease in overall ventilatory rate) (Table 5.8). Differences in the total number of beats of control and exposed animals recorded at 21°C, and in animals of the same exposure group at different temperatures, indicate that factors other than changes in ventilatory rate must be causing the differences in total hourly beat numbers (Table 5.7). For example, total ventilation volume in copper-exposed crabs at 21°C is higher than that of control animals at the same temperature, however, ventilatory rate shows a significant decrease. In this case, a more even distribution of scaphognathite rate frequencies, together with an increase in the time spent

in bilateral activity and a decrease in the time spent in bilateral and unilateral apnoea, account for the increase in total ventilation volume. In animals of the same treatment group, but at different temperatures, the discrepancies in beat rate and ventilation volume are related to the length of unilateral pauses. Periods of unilateral apnoea are significantly shorter in control animals at 15°C than 21°C. A decrease in the amount of time spent in bilateral scaphognathite activity, and an increase in the time spent in bilateral apnoea contribute to the reduction in total beat numbers. Temperature affects on these aspects (time spent in bilateral activity, duration of unilateral and bilateral apnoea periods) of ventilatory behaviour of copper-exposed animals were different from control crabs. In the case of bilateral activity, interaction between copper and temperature is highly significant. In response to the rise in temperature, periods spent in bilateral scaphognathite activity in exposed animals are, therefore, lengthened and periods of unilateral and bilateral apnoea shortened. These trends are supported by the relative contributions made by left and right scaphognathites to the total ventilatory effort. The rise in temperature resulted in a more equal relationship between the activity levels of the two sides in copper-exposed animals (Fig. 5.15), a trend also present in control animals. These results illustrate that changes in scaphognathite beat rate are not the only means of altering ventilation volumes in *C. maenas*, but that adjustments in the activity relationships between left and right scaphognathites are equally important.

In general, control and exposed animals displayed opposite reactions to temperature increase, which implies that changes in physiological response are caused by exposure to copper. These shifts in physiological response are likely to be related to structural damage at the exchange surfaces of the gills. It cannot be ruled out, however, that these changes in scaphognathite activity are only partly a reflection of physiological compensation, but also caused by the direct toxic action of copper on neuronal control mechanisms (Waldichuck, 1974).

Although many of the observed differences in the scaphognathite activity of control and copper-exposed *C. maenas* are not significant, their additive effects may lead to differences in ventilation volume and overall ventilatory rate.

CAPMON data recordings do not allow the distinction between forward or reversed scaphognathite beating and results presented here are a reflection of total ventilatory activity, combining forward and reversed movements. It is possible that some of the changes in rate measurements and total hourly beat numbers are influenced by changes in the frequency and duration of reversals. In the resting respiratory physiology of

control *C. maenas*, reversals account for < 8% of the time spent in ventilatory activity (with a frequency of 18 reversals per hour, each lasting for approximately 3 seconds) (Cumberlidge and Uglow, 1977a). Reversals are, therefore, unlikely to have a significant effect on results. Taylor *et al.* (1973) observed an increase in the frequency of reversals in *C. maenas* when the environmental temperature was raised from 6°C to 17°C, however, the duration of reversals simultaneously decreased, resulting in no change in the total time spent in ventilatory reversal. Exposure of *C. maenas* to very high temperatures, just below the threshold temperature which causes "emigration" from the water (*c.* 28°C), resulted in a significant increase of reversal frequency and duration (Taylor and Wheatly, 1979). The experimental temperatures chosen for this study (15°C and 21°C) are considerably lower, and thus unlikely to cause such drastic changes in reversal patterns.

In summary, the 10 day exposure to 500 μg Cu L^{-1} resulted in severe ultrastructural gill damage in *C. maenas*, affecting especially the anterior respiratory gills. This cellular damage occurred despite a significant increase in the concentrations of metallothionein within the gill tissue. Temperature increase affects the ventilatory behaviour of control and copper-exposed crabs in different ways (Table 5.7). In control animals, the number of scaphognathite beats (per hour) declined, although the beat rate increased and a more equal relationship in the activity levels of left and right scaphognathites was established. These changes are based on a reduction of the time spent in bilateral ventilatory activity and an increase in the duration of bilateral and unilateral periods of apnoea and frequency of the latter. On the other hand, exposed animals responded to the rise in temperature with an increase in the number of scaphognathite beats (per hour) and a more equal relationship between activity levels of left and right scaphognathites. Respiratory rate did not move in a particular direction (i.e. faster or slower), but became more varied. These changes were supported by an increase in bilateral scaphognathite activity and frequency of unilateral periods of apnoea, and a reduction in the duration of bilateral and unilateral ventilatory pauses.

At 15°C, exposure to copper resulted in a decrease in the number of scaphognathite beats (per hour), concomitant with a reduction in overall ventilatory rate. The decrease in the number of scaphognathite beats is consistent with a decrease in the amount of time spent in bilateral scaphognathite activity, as well as increases in the duration of bilateral and unilateral periods of apnoea. At 21°C, exposure to copper resulted in an increase in the number of scaphognathite beats (per hour) and a decrease in ventilatory rate. The increase in the number of scaphognathite beats is based on an increase in the time spent in bilateral

scaphognathite activity, a decline in the duration of bilateral and unilateral ventilatory pauses, and no change in the frequency of the latter. In general, copper-exposed animals responded to temperature increase in the opposite manner to control animals. Changes in ventilatory activity may, therefore, represent physiological compensatory reactions, or a failure of the animals to cope with the added stress of an increase in temperature, possibly caused by the neurotoxicity of copper. Results illustrate further that changes in the activity relationships between scaphognathites are equally important in ventilation than overall changes in beat rate.

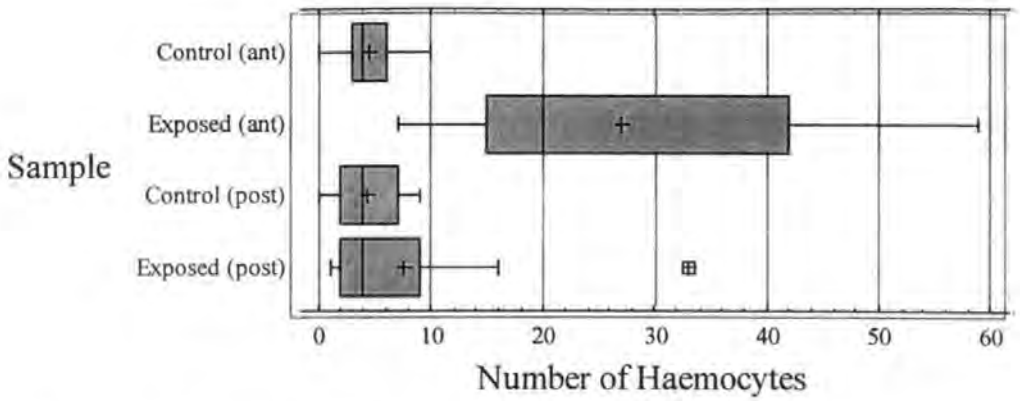


Figure 5.1: Box-Whisker plot* of the number of haemocytes present per 300 μm of lamella, in anterior (ant) and posterior (post) gill tissue of control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas*. [n=13 for anterior gills; n=14 for posterior gills]

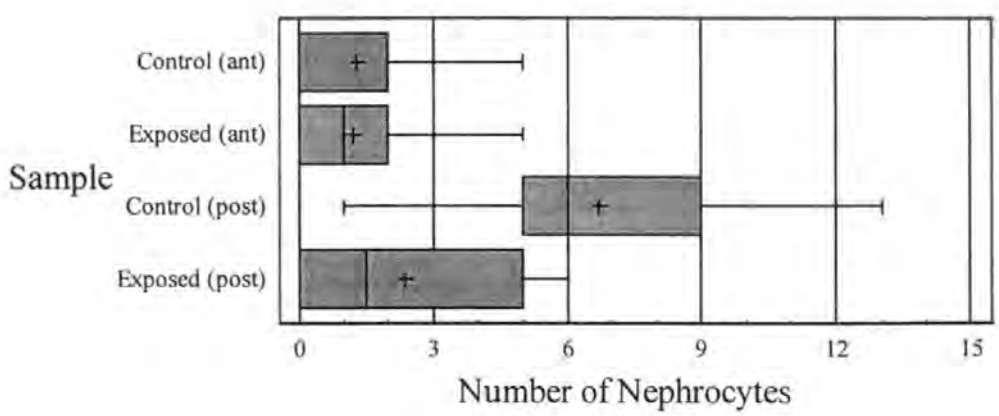


Figure 5.2: Box-Whisker plot* of the number of nephrocytes present per 300 μm of lamella, in anterior (ant) and posterior (post) gill tissue of control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas*. [n=13 for anterior gills; n=14 for posterior gills]

*see Appendix 3

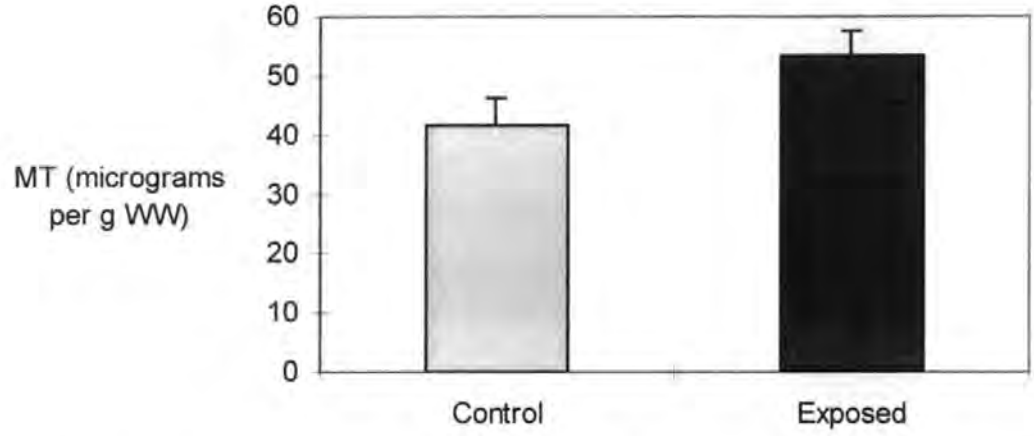


Figure 5.3: Metallothionein concentrations in μg g^{-1} wet weight (WW) in gill tissue from control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* (means and standard errors). [n (Control)=15; n (Exposed)=12]

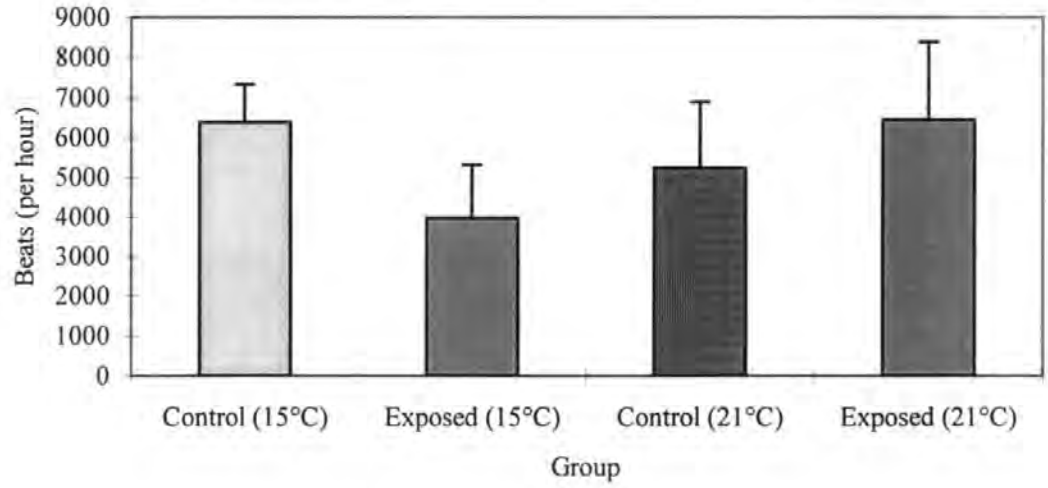


Figure 5.4: Total scaphognathite beats for the control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C (means and standard errors). Total values are made up of the sums of left and right scaphognathite beats in one hour. [n (Control/15°C)=7, n (Control/21°C)=8, n (Exposed/15°C)=8, n (Exposed/21°C)=6]

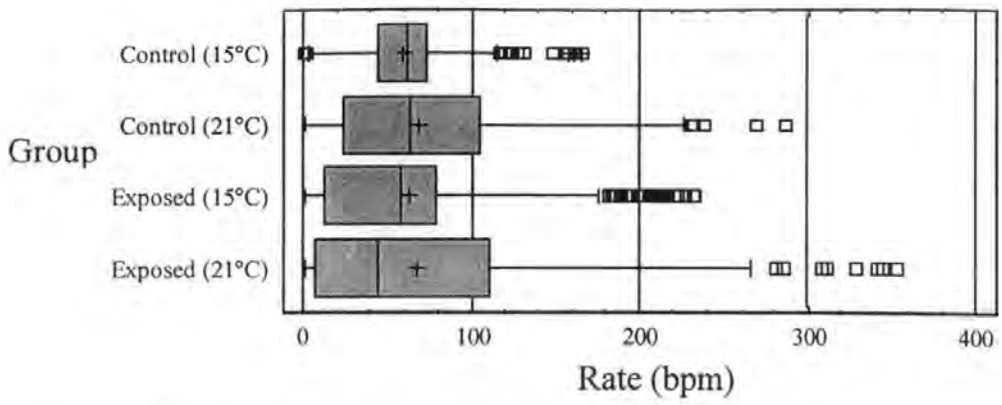


Figure 5.5: Box-Whisker plot* of scaphognathite beat rates in beats per minute (bpm) in control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C. [n (Control/15)=7, n (Control/21)=8, n (Exposed/15)=8, n (Exposed/21)=6]

*see Appendix 3

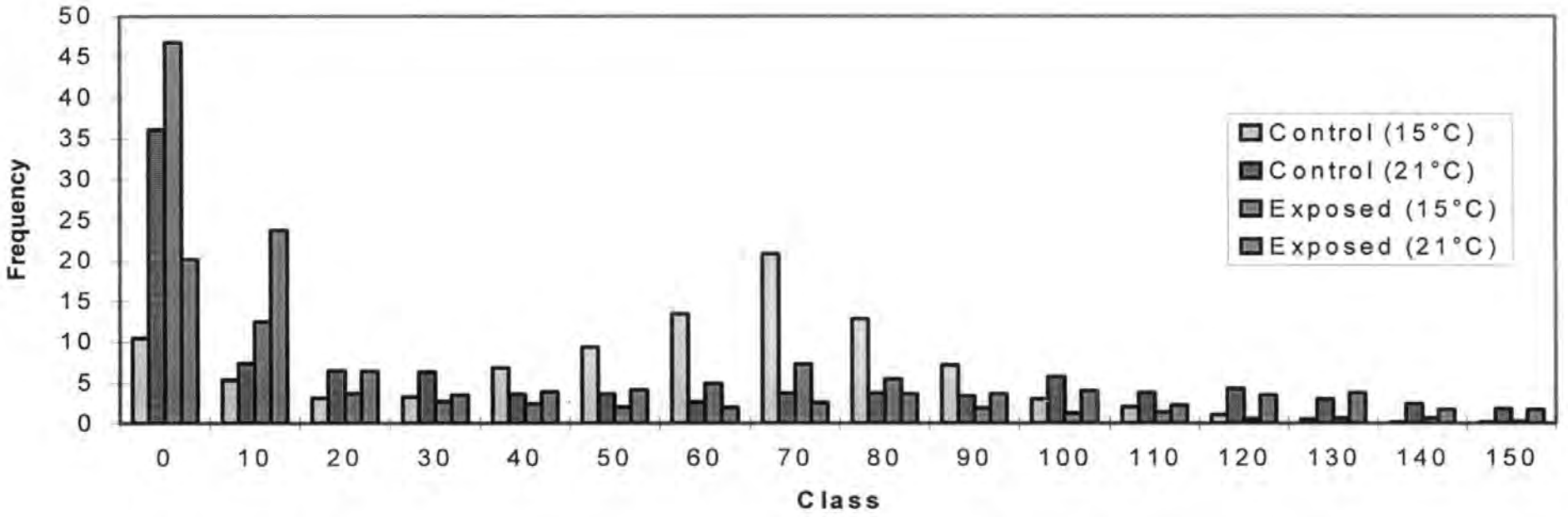


Figure 5.6a: Relative frequencies of scaphognathite beat rates in beats per minute in control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C. Classes boundaries correspond to the maximum beat rate included. [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

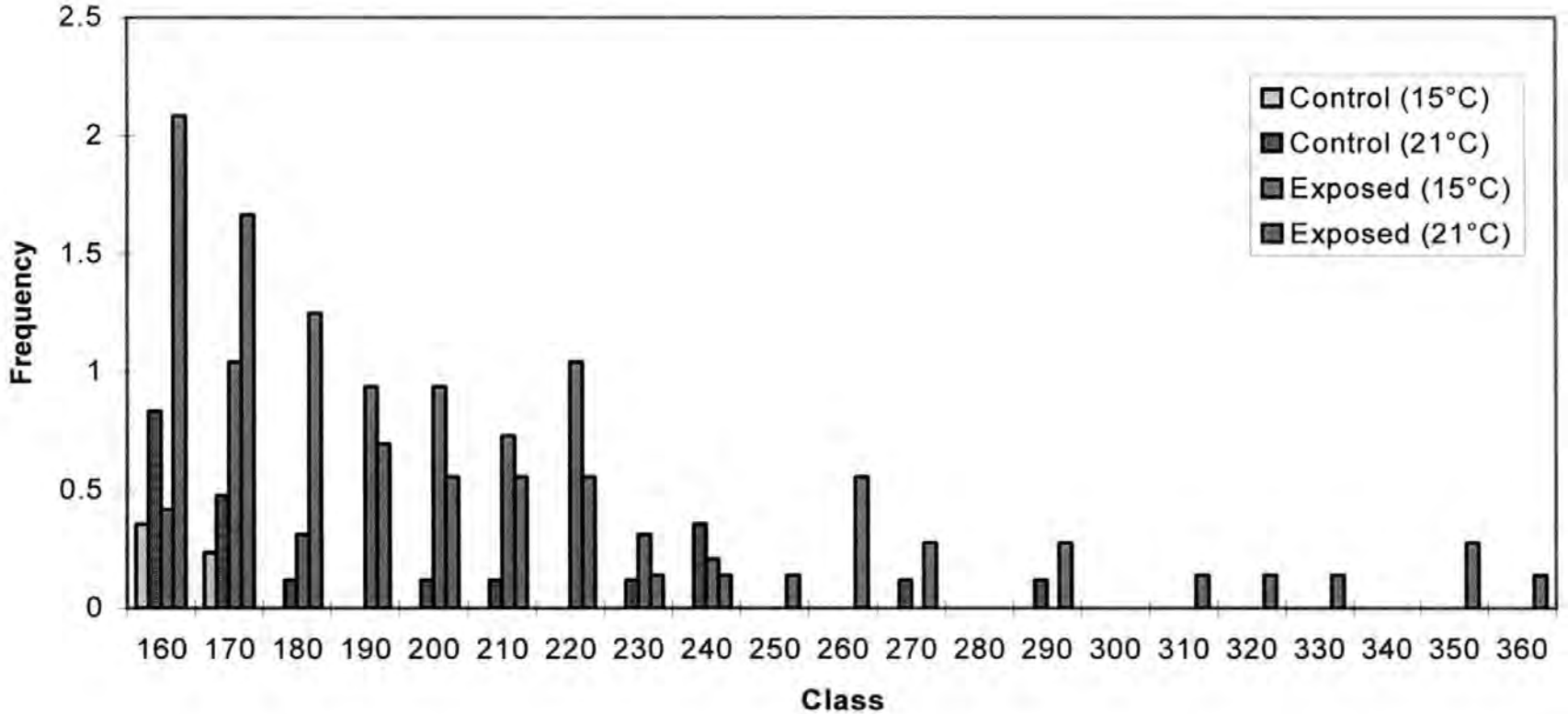


Figure 5.6b: Relative frequencies of scaphognathite beat rates in beats per minute in control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C. Class boundaries correspond to the maximum beat rate included. Note change in scale of y-axis (Frequency). [n (Control/15°C)=7; n (Control/21°C)=7; n (Expose/15°C)=8; n (Exposed/21°C)=6]

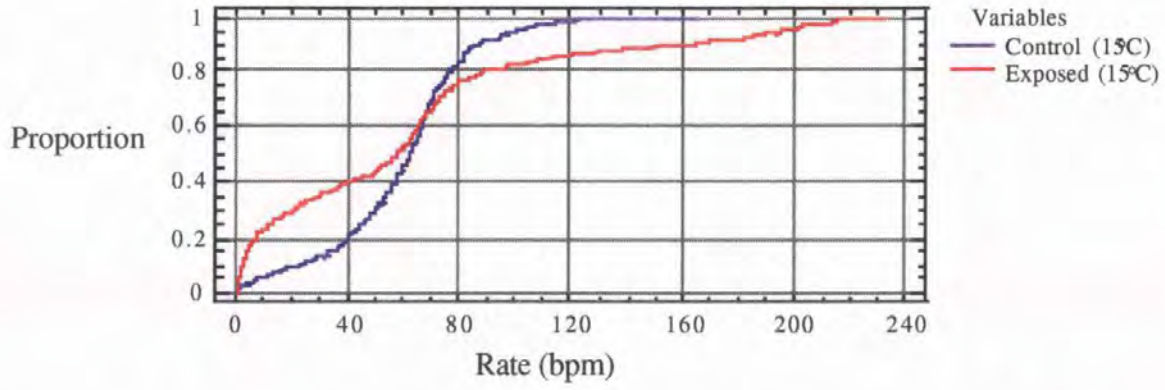


Figure 5.7: Cumulative frequency plot of scaphognathite rates in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C. [n (Control/15°C)=7; n (Exposed/15°C)=8]

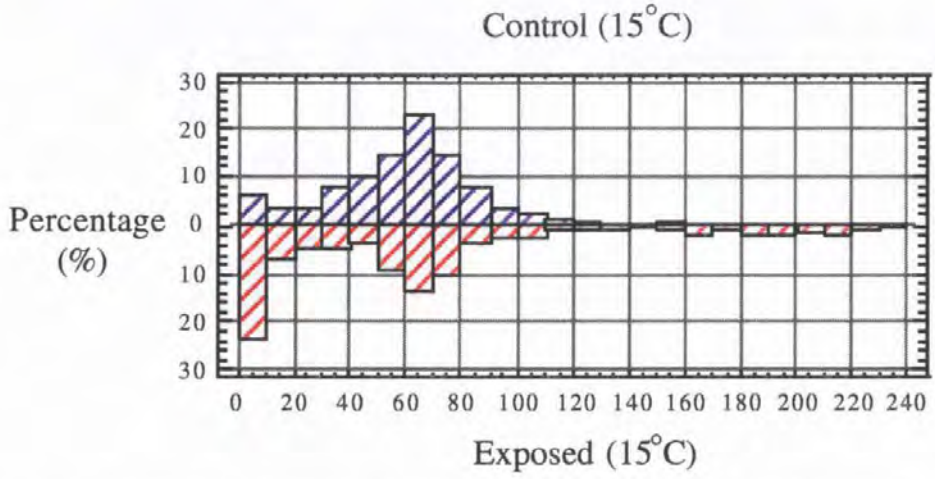


Figure 5.8: Frequency histogram of scaphognathite beat rates (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C. [n (Control/15°C)=7; n (Exposed/15°C)=8]

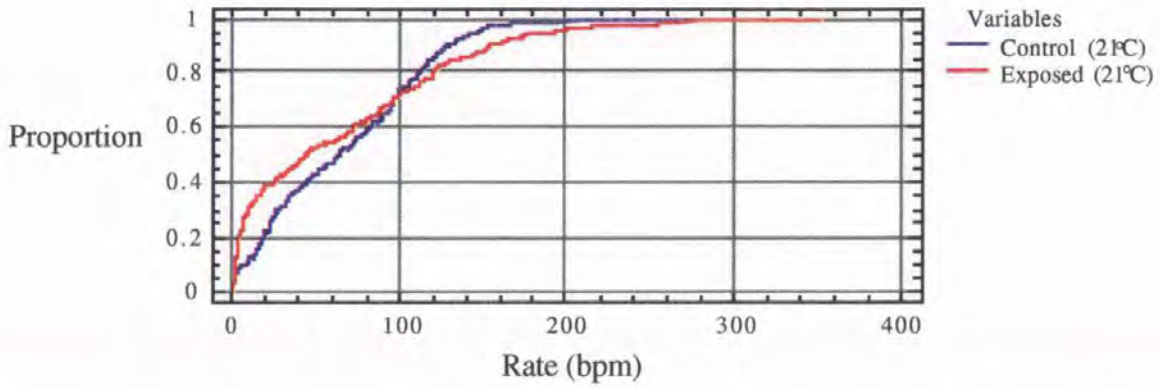


Figure 5.9: Cumulative frequency plot of the scaphognathite rates in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* at 21°C. [n (Control/21°C)=7; n (Exposed/21°C)=6]

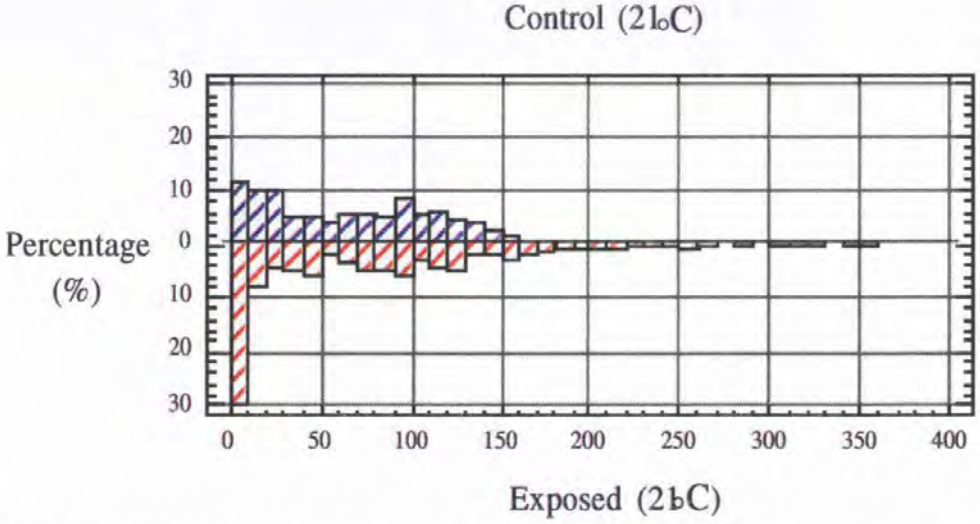


Figure 5.10: Frequency histogram of scaphognathite beat rates in beats per minute recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* at 21°C. [n (Control/21°C)=7; n (Exposed/21°C)=6]

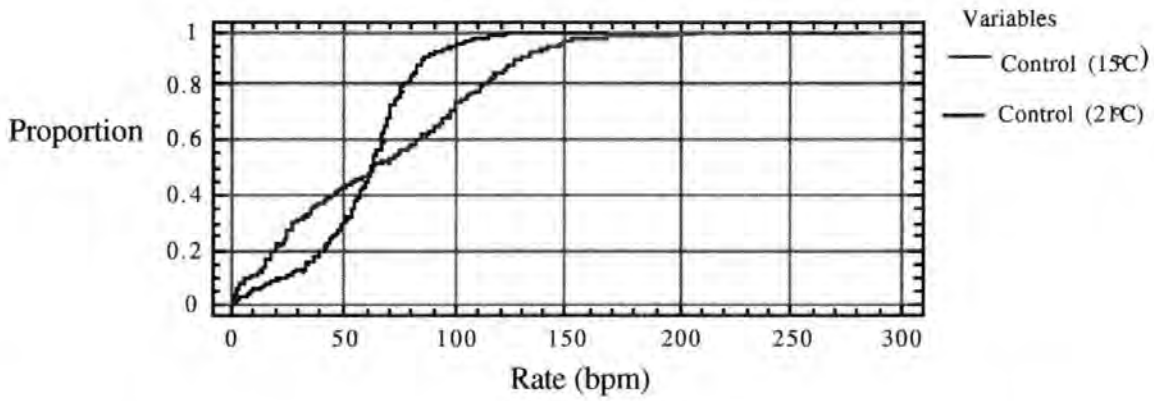


Figure 5.11: Cumulative frequency plot of scaphognathite beat rates in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control *Carcinus maenas* at 15°C and 21°C. [n (Control/15°C)=7; n (Control/21°C)=7]

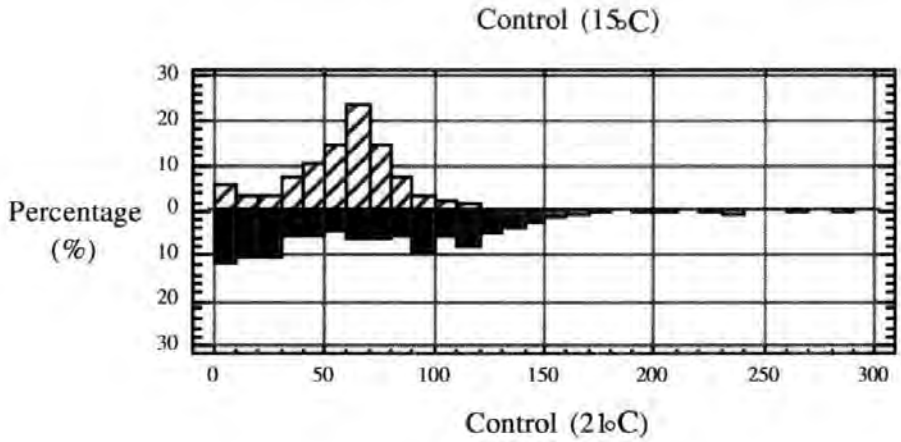


Figure 5.12: Frequency histogram of scaphognathite beat rates in beats per minute recorded over 1 h, using only active ventilatory time (>0 bpm), in control *Carcinus maenas* at 15°C and 21°C. [n (Control/15°C)=7; n (Control/21°C)=7]

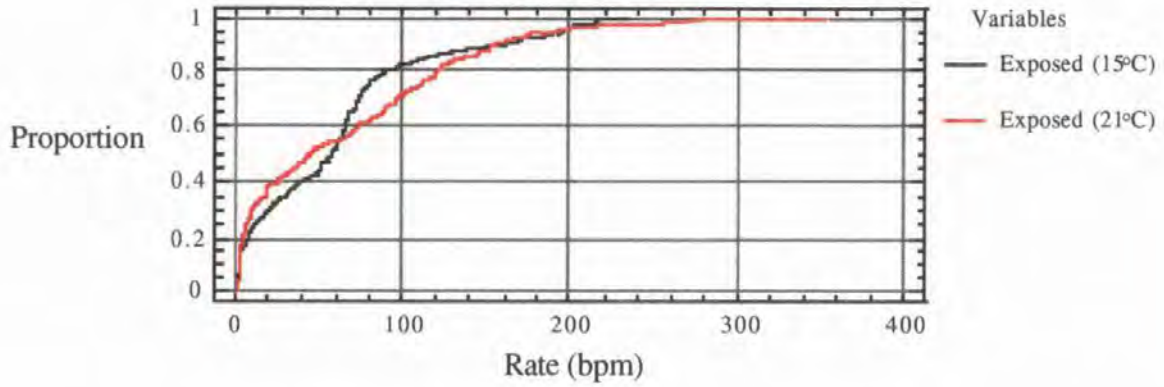


Figure 5.13: Cumulative frequency plot of scaphognathite rates in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in exposed (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C. [n (Exposed/15°C)=8; n (Exposed/21°C)=6]

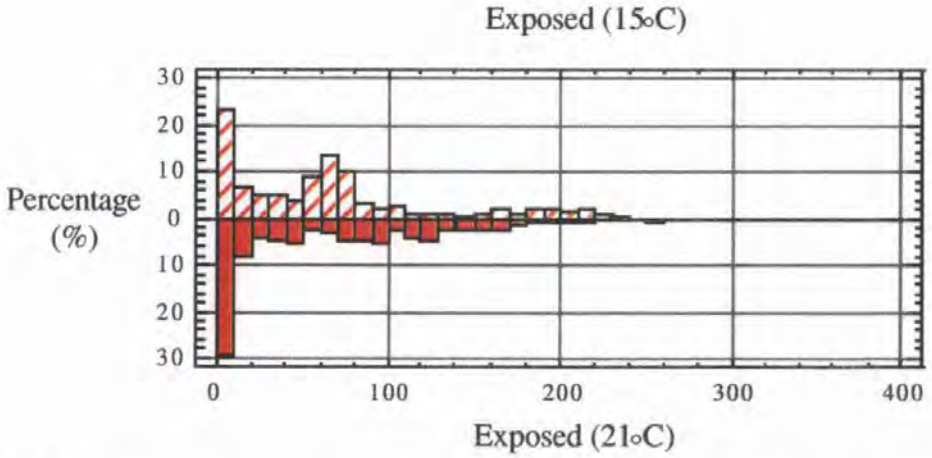


Figure 5.14: Frequency histogram of scaphognathite beat rates in beats per minute recorded over 1 h, using only active ventilatory time (>0 bpm), in exposed (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C. [n (Exposed/15°C)=8; n (Exposed/21°C)=6]

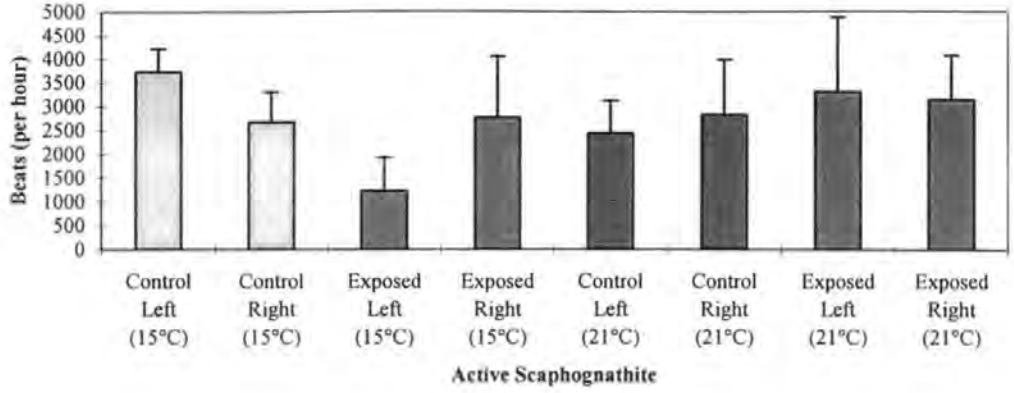


Figure 5.15: Total number of beats by left and right scaphognathites of control and exposed (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C (means and standard errors). [n (Control 15°C)=7; n (Control 21°C)=7; n (Exposed 15°C)=8; n (Exposed 21°C)=6]

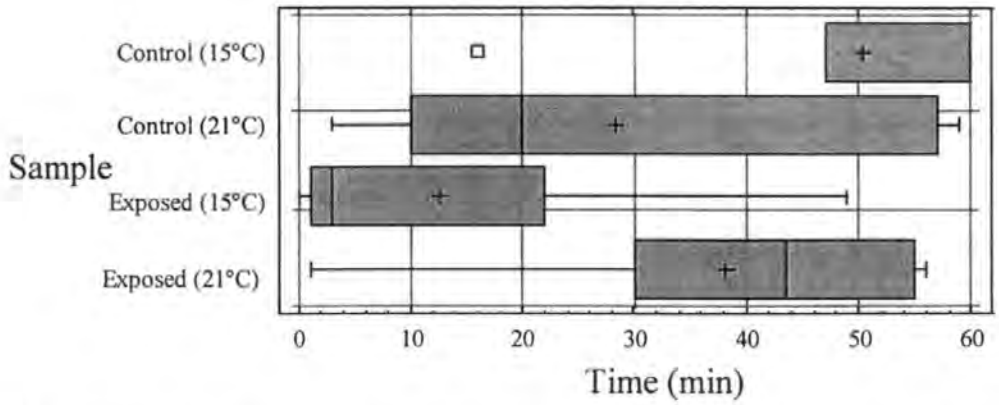


Figure 5.16: Box-Whisker plot* of the time spent in bilateral scaphognathite activity by control and exposed (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21° during the 1h monitoring period. [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

*see Appendix 3

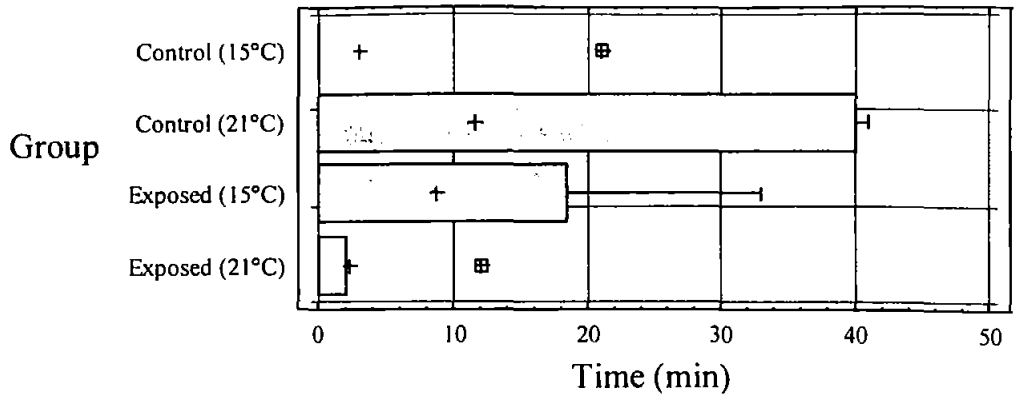


Figure 5.17: Box-Whisker plot* of the time spent in complete ventilatory rest (bilateral apnoea) by control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C. [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

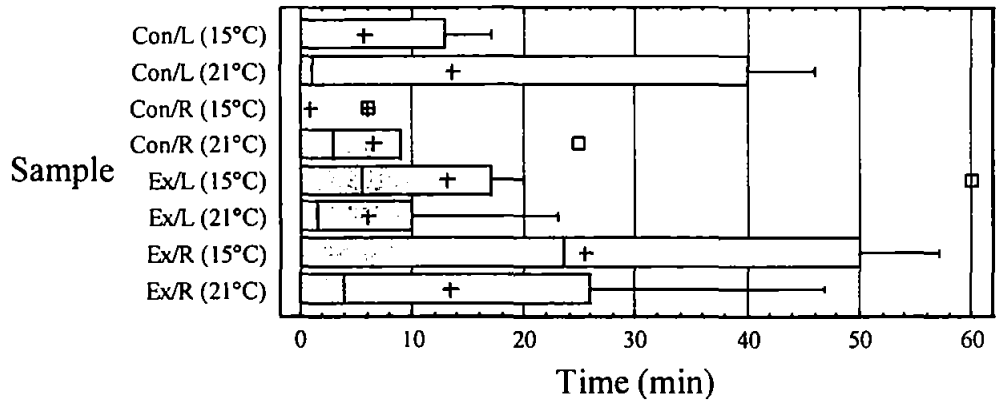


Figure 5.18: Box-Whisker plot* of time spent in unilateral ventilatory activity by control (Con) and exposed (Ex) (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C, i.e. with either left (L) or right (R) scaphognathite active. [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

*see Appendix 3

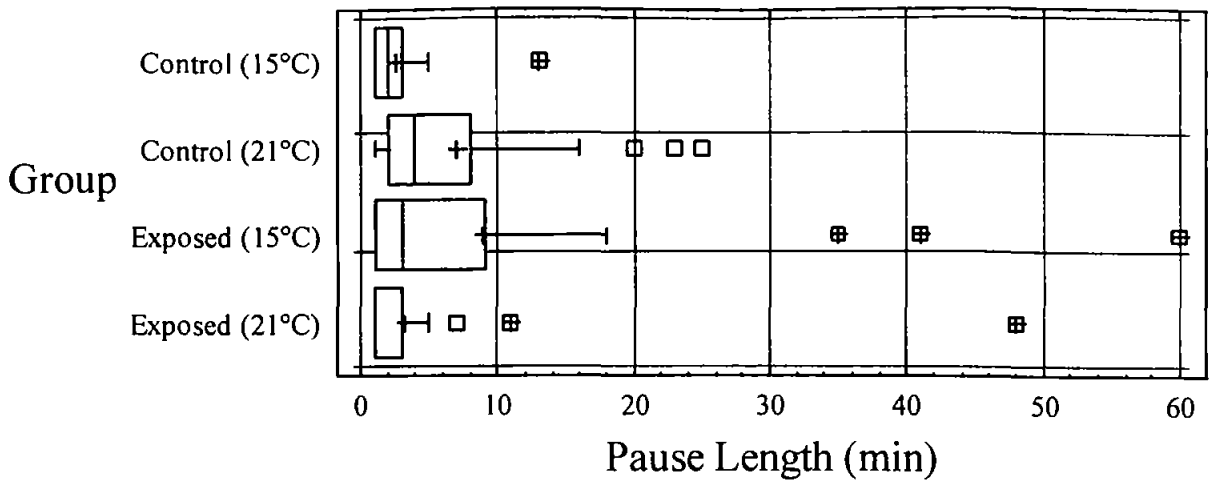


Figure 5.19: Box-Whisker plot* of the ventilatory pause lengths (apnoea) in control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15° and 21°C. [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

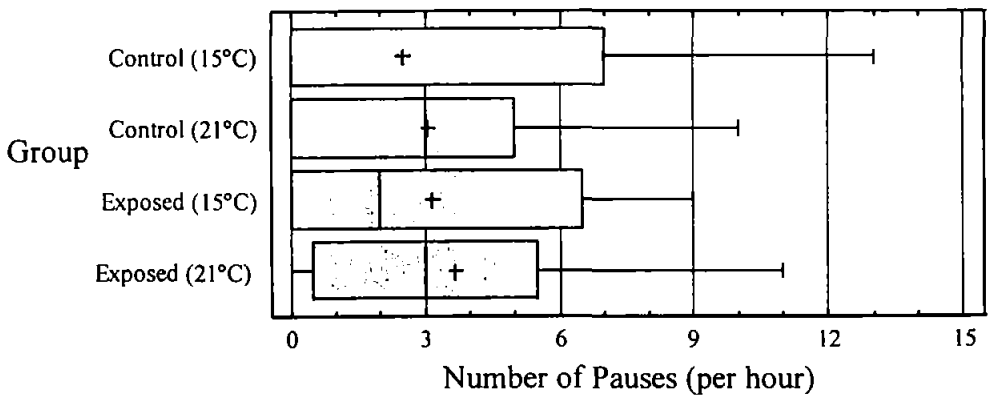


Figure 5.20: Box-Whisker plot* of the number of unilateral ventilatory pauses (apnoea) in one hour in control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15° and 21°C. [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

*see Appendix 3

Table 5.1: Significance test results of comparisons of scaphognathite beat rates in control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15° and 21°C, using Mann-Whitney Two-sample Comparisons (NS, not significant [p>0.05]; *, significant; ***, highly significant). Actual median beat rates (in beats per minute) are 62 (Control/15°C), 64 (Control/21°C), 58 (Exposed/15°C) and 45 (Exposed/21°C). [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

Comparison	Test Statistic (W)	p-Value
Control (15°C) > Exposed (15°C)	173745.0	0.0019***
Control (21°C) > Exposed (21°C)	139870.0	0.0033***
Control (15°C) < Control (21°C)	216732.0	0.0122*
Exposed (15°C) ≠ Exposed (21°C)	148767.0	0.7193 (NS)

Table 5.2: Results of the Kolmogorov-Smirnov (K-S) Two-sample Tests comparing the data distributions of scaphognathite rates of control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas*, including significance levels, maximum distance between cumulative frequency plots (DN) and the K-S test statistic (***, highly significant). [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

Comparison	DN	K-S Statistic	Significance Level
Control (15°C) ≠ Exposed (15°C)	0.23	4.01	p<0.005***
Control (21°C) ≠ Exposed (21°C)	0.21	3.45	p<0.005***
Control (15°C) ≠ Control (21°C)	0.29	5.15	p<0.005***
Exposed (15°C) ≠ Exposed (21°C)	0.15	2.50	p<0.005***

Table 5.3: Significance values and F-ratios for comparisons of the relative contributions made by left and right scaphognathites of control (C) and exposed (Ex) (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* to the total number of beats per hour at 15°C and 21°C (NS, not significant [p>0.05]) (One-way ANOVA). [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

Comparison	Test Statistic (F)	p-Value
Left C/15°C - Right C/15°C	1.72	0.2147 (NS)
Left C/21°C - Right C/21°C	0.04	0.8479 (NS)
Left Ex/15°C - Right Ex/15°C	1.1	0.3128 (NS)
Left Ex/21°C - Right Ex/21°C	0.1	0.9307 (NS)

Table 5.4: Significance values and F-ratios for the effects of the factors temperature (15°C→21°C) and copper (500 μg Cu L^{-1} for 10 days) on total beats per hour by left and right scaphognathites in *Carcinus maenas* (NS, not significant [p>0.05]) (Multifactor ANOVA, 2-way Interaction). [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

Factor	Test Statistic (F-Ratio)	p-Value
Left Scaphognathite		
Temperature	0.19	0.6667 (NS)
Copper	0.82	0.3744 (NS)
Interaction	3.59	0.0704 (NS)
Right Scaphognathite		
Temperature	0.06	0.8062 (NS)
Copper	0.04	0.8452 (NS)
Interaction	0.01	0.9155 (NS)

Table 5.5: Significance values and Mann-Whitney test statistics (W) for comparisons of median time spent in bilateral scaphognathite activity by control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C, using Mann-Whitney Two-sample Comparisons (NS, not significant [p>0.05]; *, significant; ***, highly significant. [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

Comparison	Test Statistic (W)	p-Value
Control (15°C) > Exposed (15°C)	3.0	0.0021***
Control (21°C) ≠ Exposed (21°C)	24.0	0.721 (NS)
Control (15°C) >Control (21°C)	8.0	0.0194*
Exposed (15°C) <Exposed (21°C)	38.0	0.0403*
Control (15°C) ≠ Exposed (21°C)	10.5	0.1469 (NS)
Control (21°C) > Exposed (15°C)	12.0	0.0358*

Table 5.6: Results of Mann-Whitney two-sample comparisons of the length of ventilatory pauses recorded unilaterally from control and exposed (500 μg Cu L^{-1} for 10 days) *Carcinus maenas* at 15°C and 21°C (NS, not significant [p>0.05]; *, significant; ***, highly significant). [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

Comparison	Test statistic (W)	P-value
Control (15) < Control (21)	1075.0	0.0005***
Exposed (15) > Exposed (21)	704.5	0.0009***
Control (15) < Exposed (15)	1133.5	0.0182*
Control (21) > Exposed (21)	489.5	0.00007***
Control (15) ≠ Exposed (21)	674.5	0.3174 (NS)
Control (21) ≠ Exposed (15)	979.0	0.4571 (NS)

Table 5.7: Summary of the effect of increased temperature on the scaphognathite activity of control (C) and exposed (Ex) (500 µg Cu L^{-1} for 10 days) *Carcinus maenas* (NS, not significant [$p>0.05$]; *, significant [$p<0.05$], ***, highly significant, [$p<0.005$]). [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

The Effect of increased Temperature (15°C - 21°C) on Scaphognathite Activity in *Carcinus maenas*.	
Control animals	**Exposed animals**
Total Scaphognathite Beats (per hour)	
C/15 > C/21 (NS)	Ex/15 < Ex/21 (NS)
Beat Rate (beats per minute)	
C/15 < C/21*	Ex/15 ≠ Ex/21 (NS)
Frequency Distributions	
C/15 ≠ C/21*** C/15: large peak at 51-80 bpm C/21: even distribution of data	Ex/15 ≠ Ex/21*** Ex/15: small peak at 51-80 bpm large peak at 1-10 bpm Ex/21: large peak at 1-10 bpm
Contributions of left and right scaphognathites	
p=0.2147 (15°C) p=0.8479 (21°C)	p=0.3128 (15°C) p=0.9307 (21°C)
Bilateral Activity	
C/15 > C/21*	Ex/15 < Ex/21*
Bilateral Apnoea	
C/15 < C/21 (NS)	Ex/15 > Ex/21 (NS)
Unilateral Apnoea (frequency)	
C/15 ≠ C/21 (NS)	Ex/15 ≠ Ex/21 (NS)
Unilateral Apnoea (duration)	
C/15 < C/21***	Ex/15 > Ex/21***

Table 5.8: Summary of the effect of copper (500 μg Cu L^{-1} for 10 days) on the scaphognathite activity of *Carcinus maenas* at two different temperatures (15°C and 21°C) (NS, not significant [p>0.05]; *, significant [p<0.05];**, very significant [p<0.01]; ***, highly significant, [p<0.005]). [n (Control/15°C)=7; n (Control/21°C)=7; n (Exposed/15°C)=8; n (Exposed/21°C)=6]

The Effect of Copper on Scaphognathite Activity in *Carcinus maenas* at two different Temperatures (15°C and 21°C)	
15°C	**21°C**
Total Scaphognathite Beats (per hour)	
C/15 > Ex/15 (NS)	C/21 < Ex/21 (NS)
Beat Rate (beats per minute)	
C/15 > Ex/15***	C/21 > Ex/21***
Frequency Distributions	
C/15 ≠ Ex/15*** C/15: large peak at 51-80 bpm Ex/15: small peak at 51-80 bpm large peak at 1-10 bpm	C/21 ≠ Ex/21*** C/21: even distribution of data Ex/21: large peak at 1-10 bpm
Contributions of left and right scaphognathites	
p=0.2147 (C) p=0.3128 (Ex)	p=0.8479 (C) p=0.9307 (Ex)
Bilateral Activity	
C/15 > Ex/15**	C/21 < Ex/21 (NS)
Bilateral Apnoea	
C/15 < Ex/15 (NS)	C/21 > Ex/21 (NS)
Unilateral Apnoea (frequency)	
C/15 ≠ Ex/15 (NS)	C/21 ≠ Ex/21 (NS)
Unilateral Apnoea (duration)	
C/15 < Ex/15*	C/21 > Ex/21***

CHAPTER 6

EFFECTS OF COPPER AND HYPOXIA ON SCAPHOGNATHITE ACTIVITY IN *CARCINUS MAENAS*, WITH REFERENCE TO GILL ULTRASTRUCTURE AND METALLOTHIONEIN

Abstract

Exposure of *Carcinus maenas* to copper and/or hypoxia resulted in increased total numbers of scaphognathite beats (in 1 h). This increase was consistent with a rise in ventilatory rate. Exposure to copper and/or hypoxia caused a reduction in the amount of time spent in bilateral and unilateral apnoea. Exposure to copper resulted in a strong unilateral scaphognathite dominance. The latter may represent a means of reducing the metabolic cost of ventilation. The relationship between the activity levels of right and left scaphognathites became more equal during hypoxia. Simultaneous exposure to copper and hypoxia exacerbated the changes in ventilatory activity.

6.1 Introduction

The effects of hypoxia/anoxia on crustaceans have been investigated widely, including changes in behaviour, cardiac and scaphognathite activity, oxygen consumption, metabolism and haemolymph biochemistry. For example, in the crayfish *Orconectes rusticus*, hypoxia resulted in a 3-fold increase in ventilatory and cardiac rates (Wilkes and McMahon, 1982). These changes in physiological rates optimise oxygen delivery to the gill chambers and the tissues, as well as affecting respiratory alkalosis, which in turn increases haemocyanin oxygen affinity (Wilkes and McMahon, 1982). Increased scaphognathite activity, in response to reduced oxygen tensions, has also been reported for the lobster *Homarus gammarus* (Spoek, 1974), the squat lobsters *Munida rugosa* and *M. sarsi* (Zainal *et al.*, 1992), the blue crab *Callinectes sapidus* (Batterton and Cameron, 1978), and the shore crab *Carcinus maenas* (Taylor, 1976; Jouve-Duhamel and Truchot, 1985; Johnson and Uglow, 1987), although absence of any ventilatory rate changes in *C. maenas* (at the same hypoxia level) has also been reported (Taylor *et al.*, 1973). In *C. maenas*, cardiac activity remained unaffected to a critical oxygen level of 60-80 mmHg (equivalent to 36-50% oxygen saturation), beyond which bradycardia occurred simultaneously with the loss of saturation of the haemocyanin (Taylor *et al.*, 1973; Taylor, 1976). Ventilatory and cardiac responses of crustaceans to hypoxia are reviewed by Taylor (1982). In the prawns *Palaemon elegans* and *P. serratus*, hypoxia leads to an increase in haemolymph L-lactate (indicative of anaerobic metabolism) and glucose (Taylor and Spicer, 1987). In *C. maenas*, significantly increased levels of lactate have been reported with decreasing oxygen tensions (Johnson and Uglow, 1987) and during recovery from anoxic exposure, concomitant with increased rates of oxygen consumption and cardiac rate (Hill *et al.*, 1991). With regard to behaviour in shallow water, hypoxia initially causes an increase in locomotor activity of *C. maenas* (Taylor and Butler, 1973), followed by partial emergence of from the water to allow "bubbling" of air and water in the branchial chambers by scaphognathite reversal (Taylor and Butler, 1973; Taylor *et al.*, 1973).

Although the above summary illustrates the wealth of studies reporting the effects of hypoxia on various aspects of crustacean physiology and behaviour, there are few studies involving the combined effects of hypoxia and trace metals on Crustacea (Jones, 1942; Russel Hunter, 1949; Spicer and Weber, 1992; Spicer, 1995). Hypoxia is often present in combination with such contaminants, e.g. as the consequence of sewage discharges in which industrial effluent (and the trace metals it contains) and domestic sewage are frequently combined (GESAMP, 1990). Crustaceans inhabiting areas receiving

such discharges are, therefore, exposed simultaneously to decreased oxygen levels in the water (due to the decay of organic material) and trace metals from the waste discharge. This study investigates whether environmental hypoxia compounds the effects of copper on the scaphognathite activity of the shore crab *C. maenas*, a species which inhabits estuaries and coastal areas (Hayward and Ryland, 1995), the zones most affected by urbanisation and industrial activities (McLusky, 1989). Physiological measurements are considered in the context of copper-induced damage to the ultrastructure of the gills (e.g. Lawson *et al.*, 1995), and changes in the concentration of metal-binding protein (metallothionein) in gill tissues, as these organs represent one of the prime targets of water-borne trace metals (Hebel *et al.*, 1997).

6.2 Materials and Methods

6.2.1 Collection and holding conditions

In November 1996, 24 intermoult male *Carcinus maenas* with green carapaces, and of approximately equal size (carapace width *c.* 6 cm) and weight (50-60 g wet weight), were collected from the Avon Estuary, near Bantham (Devon). Collection, transport methods and holding conditions were as described in Section 3.2.3.

6.2.2 CAPMON experimental procedure

Preparation of the crabs for scaphognathite activity recordings was carried out as described in Section 3.2.4. Each crab was placed into a glass beaker. The beakers were covered with polystyrene lids, through which aeration was continued, resulting in a 90% (±3%) oxygen saturation of the water. All oxygen measurements were performed using a Strathkelvin oxygen meter (SI, Model 781). Crabs were left to acclimate to their surroundings for 1 h before scaphognathite activity was recorded. Recording time was 1 h. The animals were moved into identical beakers (handling time 5 sec), containing seawater (salinity of 35) with an oxygen saturation of 30% (±4%), achieved by bubbling with nitrogen gas. The tightly fitting polystyrene lids, as well as the high-sided glass beakers, made it impossible for the crabs to ventilate their gills by bubbling water at the surface. Due to the short handling time, acclimation time was reduced to 30 min (recording time remained at 1 h). Oxygen levels decreased by approximately 15% throughout this period, i.e. to a total oxygen saturation value of 25.5% (±4%). By using the same animals for normoxic and hypoxic scaphognathite activity measurements, each crab represented its own control.

Parametric statistical tests were applied to the data when possible. In some cases, the data were successfully transformed (Log+1) to achieve normality, and thus permit

parametric methods. When this was not possible, non-parametric statistics were applied. The effects of copper and/or hypoxia on the total number of scaphognathite beats recorded were assessed by Advanced Regression/General Linear Models. Comparisons of beat rate were made by Mann-Whitney Two-sample Tests and comparisons of the rate data distributions by Kolmogorov-Smirnov Two-sample Tests. Activity relationships between left and right scaphognathites (total beats per hour, time in unilateral activity) were described using Kruskal-Wallis Two-sample Comparisons. Data recorded for bilateral activity and bilateral apnoea were transformed (Log+1) and analysed by Advanced Regression/General Linear Models, and, in the latter case, by One-way ANOVA. Duration and frequency of unilateral apnoea periods were compared using Kruskal-Wallis Multiple-sample Comparisons. Differences in the frequency of pauses were investigated further by Mann-Whitney Two-sample Tests. All statistical tests were performed using *Statgraphics Plus for Windows (Version 2.1)*.

6.2.3 Ultrastructural gill analysis

At the end of the CAPMON recording, an anterior gill (number 5) was removed from the right gill chamber of two control and two copper-exposed crabs. Gill tissue could not be removed after the normoxic phase of monitoring as the same crabs were used for the hypoxic measurements. Further tissue processing for electron microscopical analysis was carried out as described in Section 2.2.

6.2.4 Metallothionein quantification

Quantification of metallothionein in the gill tissue of control and copper-exposed *C. maenas* was carried out as described in Section 3.2.6. Comparisons were made between control and copper-exposed tissues collected at the end of the CAPMON monitoring. Gill tissue was not removed after the normoxic CAPMON phase, as the crabs were used for hypoxic monitoring. (Tissue collection for metallothionein quantification requires total animal dissection.) Data were analysed by One-way ANOVA using *Statgraphics Plus for Windows (Version 2.1)*.

6.3 Results

6.3.1 Gill ultrastructure

Gill ultrastructure analysis of anterior gills taken from 2 control and 2 copper-exposed animals confirmed that the 10 days exposure to 500 μg Cu L^{-1} caused the same cellular changes as reported in Section 2.3.5 (see Fig. 2.11 for a summary). No differences due to the 90 min hypoxic exposure were evident.

6.3.2 Metallothionein

Metallothionein gill tissue concentrations were not significantly elevated following exposure to 500μg Cu L^{-1} for 10 days compared with control levels ($p=0.1852$; $F=1.88$; One-way ANOVA) (Fig. 6.1).

6.3.3 Scaphognathite activity

There was a significant effect of copper ($F=17.64$; $p=0.0004$) (Fig. 6.3) and oxygen level ($F=10.21$; $p=0.0045$) (Fig. 6.4) on the total number of scaphognathite beats in 1 h of recording (Advanced Regression/General Linear Models). Control crabs in normoxic conditions exhibited the lowest total number of beats, whereas copper-exposed animals monitored under hypoxic conditions expressed greatest total scaphognathite activity (Fig. 6.2). In addition, the median beat rate (per minute) was significantly elevated in copper-exposed compared with control animals under normoxic and hypoxic conditions, and significantly elevated by hypoxia in the control and exposed group compared with normoxic recordings (Tables 6.1 and 6.2; Fig. 6.5).

The frequency distributions of scaphognathite beat rate data (bpm; including periods of apnoea) are illustrated in Figures 6.6a and 6.6b. At both oxygen levels, control animals spent more time in ventilatory rest (i.e. class 0) than copper-exposed crabs. The time spent by control animals in apnoea was less affected by hypoxia than in copper-exposed crabs, where a noticeable decrease was recorded. Only copper-exposed animals under hypoxic conditions displayed beat rates exceeding 120 bpm, with a maximum rate of 240 bpm. Comparison of these frequency distributions showed significant differences (Table 6.3). In normoxic conditions, control animals spent more time at low beat rates, seen as a steep rise in the cumulative frequency plot compared with the more linear plot for copper-exposed animals (Fig. 6.7). The frequency histogram (Fig. 6.8) illustrates these differences in distribution, with control animals displaying a peak at scaphognathite beat rates between 1 and 10 bpm compared with a more even distribution in the data from the exposed animals in normoxic conditions. A similar relationship is shown by both groups in hypoxic conditions (Fig. 6.9), however, the exposed animals exhibited a greater range in beat rate than the control animals. In control animals, the dominant beat rate appeared to lie between 1 and 10 bpm, whereas no dominant beat rate was visible for copper-exposed animals (Fig. 6.10). The distributions of scaphognathite beat rates were significantly different in the same animals under different oxygen conditions (Figs. 6.11 to 6.14). Although the cumulative frequency curves for control animals in normoxic and hypoxic conditions have similar initial gradients, due to a similar preference for very low beat rates

(1-10 bpm), the former rises more steeply at beat rates exceeding *c.* 15 bpm (Fig. 6.11). This difference is illustrated in the frequency histogram, where a greater percentage of data lie between 21 and 30 bpm in recordings made in normoxic rather than hypoxic conditions, different rates having more equal part in the distribution in the ventilatory behaviour in the latter treatment (Fig. 6.12). A similar pattern between normoxic and hypoxic recordings is visible when the ventilatory rate data from exposed animals in normoxic and hypoxic conditions are compared. In this case, cumulative frequency plots diverge at a much lower rate and animals faced with hypoxia displayed a greater range of beat rates than crabs under normoxic conditions (Fig. 6.13). The reduced dominance of beat rates between 1 and 10 bpm in copper-exposed animals is clearly visible in Figure 6.14. In both treatments, however, exposed animals show a second peak at beat rates between 40 and 50 bpm, whereas the only peak in control animals lies between 1 and 10 bpm (Fig. 6.12).

The relative contributions made by left and right scaphognathites to the total number of beats recorded for control and exposed animals under normoxic and hypoxic conditions are illustrated in Figure 6.15. For control crabs, the median number of beats recorded for left and right scaphognathites were not significantly different under normoxic (test statistic=0.04; p=0.8434) and hypoxic (test statistic=2.49; p=0.1148) conditions (Kruskal-Wallis Two-sample Comparisons). For copper-exposed animals, however, the number of beats recorded was significantly higher in left than right scaphognathites under normoxic (test statistic=9.33; p=0.0023) and hypoxic (tests statistic=8.16; p=0.0043) conditions (Kruskal-Wallis Two-sample Comparisons). Although some of the means and medians in Figure 6.15 indicate an increase in total beats per hour of the individual scaphognathites in hypoxic conditions compared with normoxic measurements, there were no significant differences for the left (test statistic<0.01; p=0.9737) or right (test statistic=1.25; p=0.2642) scaphognathite of the control, or left (test statistic=0.79; p=0.3754) or right (test statistic=1.11; p=0.2927) scaphognathites of the copper-exposed animals (Kruskal-Wallis Two-sample Comparisons).

The time crabs spent with either the left or right scaphognathite beating (unilateral activity) is represented in Figures 6.16 and 6.17. In control animals, the dominance of the right scaphognathite is significant only in normoxic conditions (Table 6.4). In exposed animals, the dominance of the left scaphognathite is highly significant under normoxic and hypoxic conditions (Table 6.4). The time spent beating by any single scaphognathite,

however, is not significantly affected by transfer of the animals from a normoxic to a hypoxic environment (Table 6.4).

Figure 6.18 illustrates the total number of minutes spent in bilateral ventilatory activity or bilateral apnoea by control and exposed animals during normoxia and hypoxia. Although exposure to copper or hypoxia did not significantly affect the time spent with both scaphognathites active simultaneously (Advanced Regression/General Linear Models; F=1.71, p=0.115; copper: F=0.99, p=0.3327; oxygen level: F=0.39; p=0.5419), the total number of minutes (Fig. 6.18) and mean values of these totals (Fig. 6.19) indicate that hypoxia leads to an (insignificant) increase in the bilateral activity of copper-exposed animals.

The time spent with neither scaphognathites active (bilateral apnoea) was significantly reduced in copper-exposed crabs compared with control crabs (F=17.26; p=0.0005) (Figs 6.18 and 6.20). Oxygen concentration did not significantly affect bilateral apnoea in either control or exposed crabs (Advanced Regression/General Linear Models; F=0.01; p=0.9275) (Fig. 6.20). Significance values of comparisons made of mean times spent in bilateral apnoea by One-way ANOVA are given in Table 6.5. Time data are [Log+1]-transformed to allow parametric Advanced Regression/General Linear Models analysis.

In normoxic and hypoxic conditions, the length of unilateral ventilatory pauses (apnoea) did not differ significantly between control and exposed groups (Kruskal-Wallis Multiple-sample Comparison; test statistic=7.66; p=0.0537) (Fig. 6.21). There were, however, significant differences in the number of unilateral ventilatory pauses recorded for the different groups (Kruskal-Wallis Multiple-sample Comparison; test statistic=9.82; p=0.0202) (Fig. 6.22); control animals in hypoxia paused more frequently unilaterally than exposed animals. A similar trend was observed in normoxia but the differences were not significant (Mann-Whitney Two-sample Tests; Table 6.6).

6.4 Discussion

Exposure of *C. maenas* to 500 μg Cu L^{-1} for 10 days resulted in the same ultrastructural degeneration of anterior gill tissue as described in Section 2.3.5, following an exposure period of 14 days to the same level of copper. The relatively short exposure of the control and copper-exposed animals to hypoxia did not affect gill ultrastructure. These changes suggested functional impairment of the gill epithelia, e.g. with regard to the exchange of gases across the epithelial cells.

Despite the severe ultrastructural damage observed in the respiratory gills of copper-exposed compared with control crabs, metallothionein (MT) concentrations were not elevated significantly. The absence of a significant MT response may be due to malfunction or degeneration of the organelles and cell constituents involved in MT synthesis (e.g. ribosomes and endoplasmic reticula), caused by the toxic action of the high levels of exogenous copper. This lack of MT response may be linked also to the time lag involved in MT synthesis in invertebrate tissues (Roesijadi, 1982), allowing the toxic action of copper to severely damage the tissue prior to MT induction (Engel and Brouwer, 1984). The tissue for MT quantification was extracted from the crabs after 90 min of hypoxic exposure. Considering the shortness of this hypoxic exposure time compared with the overall period of copper exposure, it is unlikely that hypoxia depressed the MT response. The effect of hypoxia on the ability of invertebrates to synthesise MT requires further attention.

At the physiological level, copper exposure resulted in an increase in the total number of scaphognathite beats. This implies that the total ventilation volume (not measured in the current study) was greater in copper-exposed than control animals. Previous work has established that gas transfer at the gill/haemolymph interface is impaired as a result of thickening of the epithelia, consistent with lowered arterial haemolymph oxygen pressure following 4 days of exposure to 500 μg Cu L^{-1} (Nonnotte *et al.*, 1993). Increased scaphognathite beating may represent a compensatory response, i.e. resulting in a greater ventilatory volume and proportionally more oxygen arriving at the gills. Indeed, such an inferred increased gill ventilation when coupled with gill ultrastructural recovery may be prerequisites for the improvement in haemolymph oxygenation observed by Nonnotte *et al.* (1993).

Hypoxia had similar effects on the total number of scaphognathite beats in *C. maenas* as exposure to copper. Increase in the number of scaphognathite beats under hypoxia is consistent with a compensatory reaction to reduced oxygen delivery to the gills by increasing the ventilatory flow. This increase in ventilatory flow is well documented for *C. maenas* (Taylor, 1976; Taylor, 1982; Jouve-Duhamel and Truchot, 1985; Johnson and Uglow, 1987). Exposure to hypoxia, however, exacerbates the effect of copper on the number of scaphognathite beats. The percentage increase in the mean total number of scaphognathite beats of copper-exposed animals was greater than that of control animals in response to hypoxic conditions (44% and 29% respectively). Under hypoxia, copper-exposed animals appear to be compensating for the reduced oxygen delivery to the gill

surfaces by the ventilatory current and the impaired transfer of available oxygen through the damaged gill epithelia. In general, however, the increase in beat rate and total number of beats does not reach levels measured by other workers in response to hypoxia (Taylor, 1976; Taylor, 1982; Jouve-Duhamel and Truchot, 1985; Johnson and Uglow, 1987). This may be due to a shift of the critical oxygen value, beyond which ventilatory activity decreases following the initial increase. There is evidence that exposure to copper may result in such a shift (Spicer, pers. com.).

The relatively greater number of scaphognathite beats in copper-exposed *C. maenas* in normoxia compared to control animals in normoxia, and animals in hypoxic rather than normoxic conditions, is mainly due to higher scaphognathite beat rates, but is also consistent with changes in ventilatory apnoea and unilateral and bilateral scaphognathite activity (as discussed below). All experimental groups displayed beat rates within the range described as "resting level" rates (Cumberlidge and Uglow, 1977a), although scaphognathite activity levels in control animals were very low (Table 6.2). The relatively low rates in all groups are due to rates being calculated from per-minute recorded data (i.e. short periods of apnoea falling within a minute-data block are combined with scaphognathite beats to give very low rate recordings per minute). Nevertheless, the beat rates of copper-exposed animals under normoxic and hypoxic conditions were significantly higher than those of control animals. Also, under hypoxic conditions, the mean increase in rate in copper-exposed crabs was greater than in control animals (29% compared to 25%), further evidence for compensation in scaphognathite activity in response to reduced levels of oxygen in the ventilatory current and impaired transfer across the gill epithelia. During progressive hypoxia, such increases in ventilatory activity have been shown previously to maintain a constant level of oxygen delivery to the gills down to water oxygen tensions between 60 and 80 mmHg (equivalent to *c.* 36-50% oxygen saturation) (Taylor, 1976). The relative proportions of oxygen delivery to the tissues, however, from haemocyanin on the one hand, and from solution on the other, changes in favour of the latter (Taylor, 1976). These values are likely to be affected strongly by pre-exposure to copper through the structural, and thus functional, impairment of cellular transfer mechanisms. This is confirmed by the greater changes observed in terms of ventilatory rate and total ventilation volumes (theoretical) in copper-exposed than control animals, which are additionally challenged by hypoxia.

Differences in overall beat rate are caused by the dominance of either high or low-beat-rate-minutes, as highlighted in the analysis of frequency distributions. The latter also

showed that copper-exposed animals in hypoxic conditions had the highest recorded scaphognathite beat rates. This characteristic may be influenced by an increase in the duration and/or frequency of periods of rapid reversed beating as reported previously following introduction of *C. maenas* to a hypoxic environment (Taylor and Butler, 1973; Taylor, 1976). Jouve-Duhamel and Truchot (1985), however, calculated that the total time spent in reversal was not affected significantly by hypoxia.

Although the changes in overall beat rates are important components of the significant difference in the total number of scaphognathite beats recorded for control and exposed animals, the higher total count in exposed animals is also caused by a strong dominance of the left scaphognathites (in normoxic and hypoxic conditions). The dominance of either scaphognathite is considered a random feature of the ventilatory behaviour of *C. maenas* (Cumberlidge and Uglow, 1977a; Duhamel-Jouve, 1982; Taylor, 1982). The dominance of one scaphognathite in copper-exposed animals, therefore, seems surprising. Use of a single scaphognathite may offer a means of reducing the metabolic costs of ventilatory activity, i.e. the high activity of a single scaphognathite achieving a better ratio of metabolic cost to oxygen delivery/extraction, than a more equal relationship between left and right scaphognathites. Such a metabolic advantage has been suggested as the reason for the dominance of unilateral ventilatory behaviour in *Cancer magister* (McDonald *et al.*, 1977). Certainly in fish, ventilatory activity has been shown to have relatively high metabolic costs (Jones and Schwarzfeld, 1974), and data available for *Cancer magister* seem to indicate a similar requirement (Johansen *et al.*, 1970). It does, however, seem rather surprising that the left scaphognathite should be dominant in most of the copper-exposed *C. maenas*. The CAPMON equipment was sufficiently randomised during the monitoring periods, i.e. the sensors and associated computer-transducer interfaces were applied to left and right branchiostegites of control and copper-exposed animals at random. The measurements are, therefore, not influenced by equipment bias. There is, however, no neurological basis for an assumption that the neurotoxicity of copper creates sinistrality in crab ventilatory behaviour.

The total number of minutes (per hour) spent in ventilatory rest was greater in control than exposed animals, and was lower in hypoxic than normoxic conditions in both control and copper-exposed crabs. Greatest changes were observed in crabs challenged with the combined effect of copper and hypoxia. The reduction in time spent in apnoea supported the overall increase in the number of scaphognathite beats (i.e. ventilation volume) mentioned above, and is caused by a decline in the importance of bilateral apnoea

as well as the frequency of unilateral pauses in copper-exposed animals (while the duration of pauses remains unchanged). This decrease in the time spent in apnoea occurred in favour of time spent in bilateral, as well as unilateral, ventilatory activity. In general, however, apnoea is affected by copper rather than oxygen level.

Although the changes in scaphognathite activity relationships are often statistically insignificant when considered individually, their dependence on each other as well as their part in causing the significant changes at the higher level (e.g. total scaphognathite beats), makes them physiologically important.

There are many studies involving the effects of hypoxia on *C. maenas*, however, the levels of hypoxia vary, making direct comparisons with the present results difficult. In *C. maenas*, respiratory independence is maintained to a critical value of approximately 60 mmHg (equivalent to *c.* 36% oxygen saturation), at which point bradycardia sets in, coinciding with the loss of haemocyanin saturation (Taylor, 1976). The level of hypoxia chosen for the current study (*c.* 30% saturation) lies just below the critical level, and simultaneous exposure to copper and hypoxia were likely to seriously affect ventilation. Although an attempt has been made to establish threshold values required for the onset of hypoxic effects in aquatic crustaceans (Forgue *et al.*, 1992), such generalisations are easily invalidated by variations in the physiological condition of the test organisms. For example, haemocyanin concentrations (and thus the "vehicle" of haemolymph oxygen transport), vary both with moult stage (Truchot, 1978) and degree of starvation (Uglow, 1969), and crustacean haemocyanin oxygen affinity is affected by other environmental variables such as temperature and salinity (Truchot, 1973; Mauro and Mangum, 1982 a and b; Jokumsen and Weber, 1982; Burnett *et al.*, 1988). Hypoxia leads to an improved haemocyanin oxygen affinity in *C. maenas* due to an increase in lactate levels from anaerobic tissue metabolism (Truchot, 1980). In the current study, haemocyanin oxygen affinity was not measured, however, an increase in haemocyanin oxygen affinity may be partly responsible for the relatively weak response of scaphognathite activity to hypoxia. This weak response would be supported by an increase in gill perfusion, as an increase in cardiac activity is a common response to moderate hypoxia (e.g. Taylor and Butler, 1973; Wilkes and McMahon, 1982), although bradycardia occurs during extreme hypoxia (Taylor, 1976). In addition, increased branchial ventilation and perfusion facilitate the removal of dissolved carbon dioxide from the haemolymph of *C. maenas*. The resulting alkalosis increases haemocyanin oxygen affinity via the Bohr effect (Wilkes and McMahon, 1982). Exposure to copper, however, indirectly causes a decrease in oxygen affinity due to haemolymph

acidosis promoted by a Bohr shift (Truchot and Boitel, 1992). At the same time, oxygen concentration of the seawater below the critical level (as applied in the current study) leads to oxygen-unsaturated haemocyanin. At exposure levels higher than 500 μg Cu L^{-1}, the effect of haemolymph acidosis is counteracted by a simultaneous increase in lactate concentration (Nonnotte *et al.*, 1993). Exogenous copper does not directly affect oxygen-haemocyanin binding, as inorganic ions and oxygen bind to different sites at the haemolymph protein (Truchot and Boitel, 1992). Although nothing is known of the combined effects of hypoxia and copper exposure on haemocyanin oxygen affinity in *C. maenas*, haemolymph hypoxia caused by tissue damage, compounded by environmental hypoxia, may result in a strong lactic response, thus increasing oxygen affinity. This would certainly be of great physiological advantage to the impacted animals, as a greater ventilation volume coupled with improved oxygen uptake from the external medium by haemocyanin would combat the impairment of respiratory exchanges due to structural damage of the gill epithelia.

In summary, exposure of *Carcinus maenas* to 500 μg Cu L^{-1} for 10 days lead to severe ultrastructural damage of the respiratory gills. Short-term hypoxic exposure did not affect the gill ultrastructure of control or copper-exposed crabs. The ultrastructural gill damage implies impairment of gas exchange at the gills, which appeared to be compensated for by increased scaphognathite activity (see Table 6.7 for a summary). Copper-exposed animals spent significantly less time in bilateral and unilateral apnoea than control crabs. Copper-exposed crabs exhibited a marked dominance of one scaphognathite, which may represent a means of reducing the metabolic costs of respiration. In general, hypoxic conditions exacerbated the effects of copper on the ventilatory activity of *C. maenas,* including significant increases in scaphognathite rate and, therefore, the total number of scaphognathite beats. The rise in scaphognathite beat numbers (and, therefore, ventilation volume) and rate was consistent with an (insignificant) increase in the time spent in bilateral ventilatory activity and more equal relationships between left and right scaphognathites. The cause for these changes is thought to lie in the necessity to compensate for impaired oxygen transfer at the damaged gill epithelia, as well as the decline in oxygen delivery to the gill surfaces during hypoxia, by increasing the flow of water past the gill surfaces.

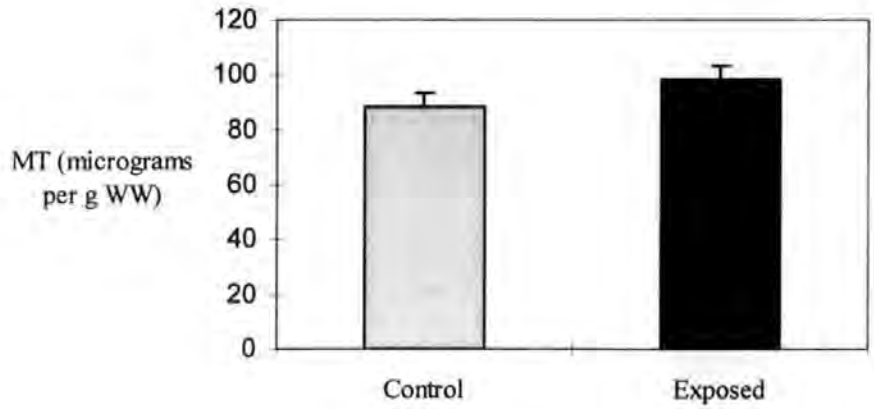


Figure 6.1: Metallothionein (MT) concentrations in gill tissue from control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in μg MT per g wet weight (WW) of gill tissue (means and standard errors). [n (control)=12; n (exposed)=11]

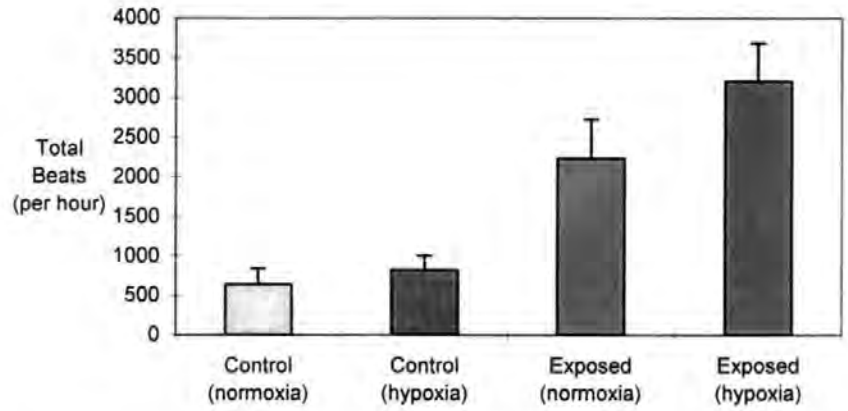


Figure 6.2: Total scaphognathite beats (in 1 h) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic and hypoxic conditions (means and standard errors). [n=11 for all groups]

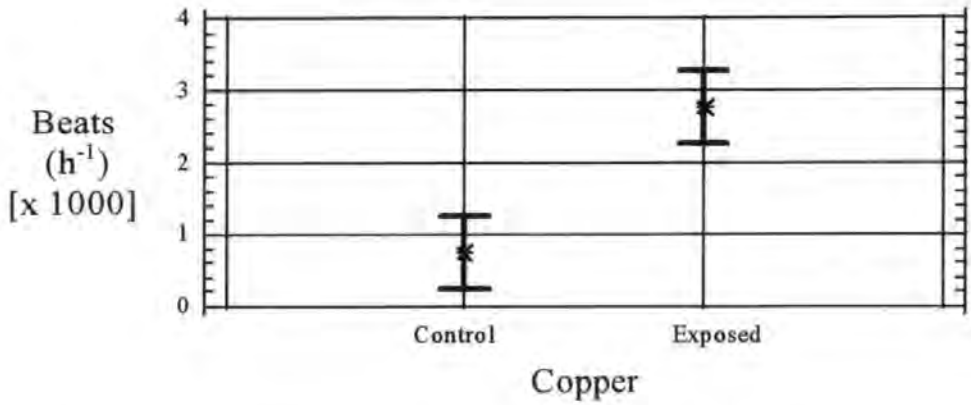


Figure 6.3: The effect of copper (10 days at 500 μg Cu L^{-1}) on the total number of scaphognathite beats (per hour) in *Carcinus maenas* (means and least-significant-difference intervals). [n=11(22)† for both groups]

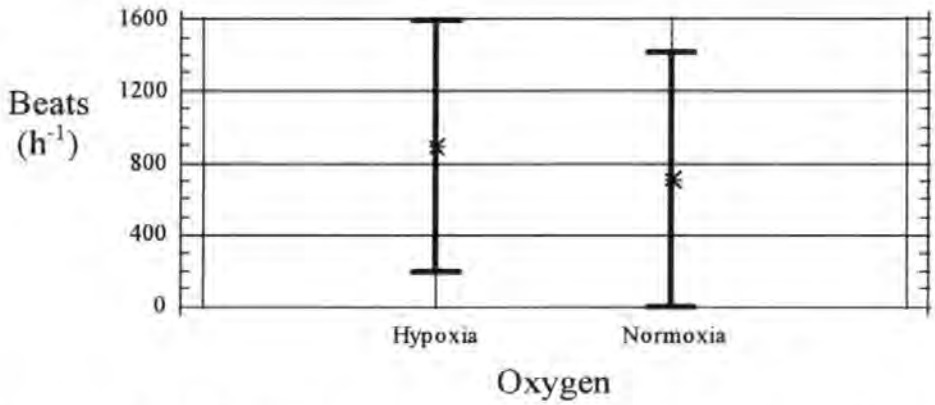


Figure 6.4: The effect of oxygen level on the total number of scaphognathite beats (per hour) in *Carcinus maenas* (means and least-significant-difference intervals). [n=22† for both groups]

†The same control or exposed animals are used in hypoxia and normoxia, i.e. used as their own control in terms of oxygen level. N-numbers are, therefore, different for the effect of copper and the effect of oxygen.

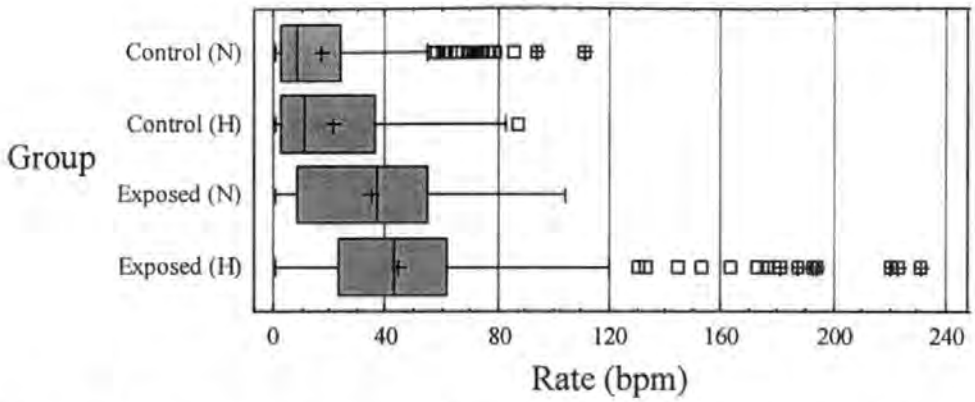


Figure 6.5: Box-Whisker plot* of scaphognathite beat rates in beats per minute (bpm) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions. [n=11 for all groups]

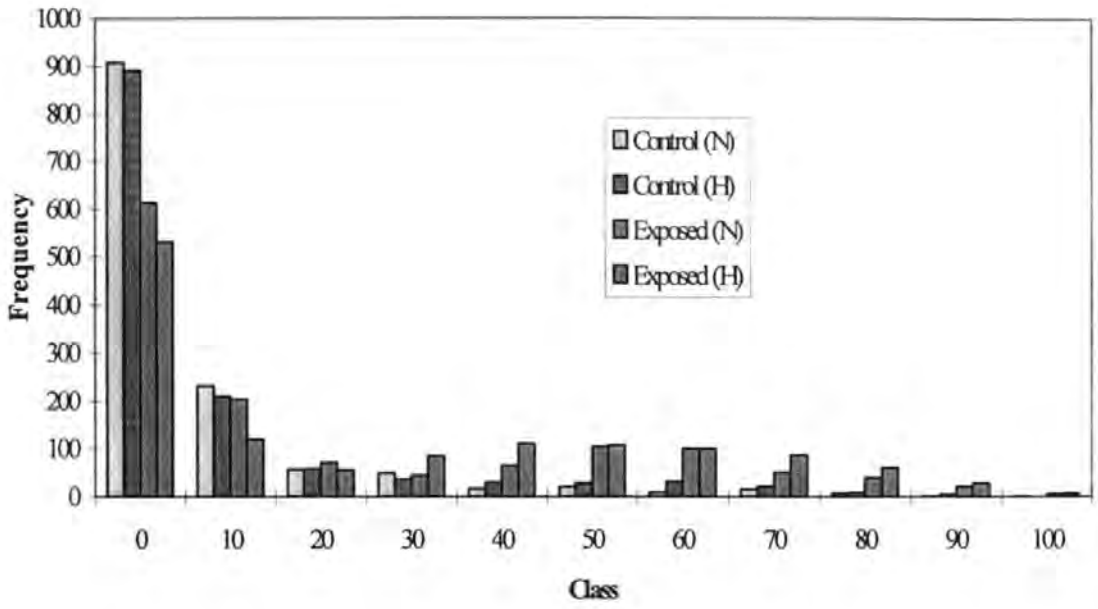


Figure 6.6a: Absolute frequencies of different scaphognathite beat rates (bpm) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions. Class boundaries given correspond to the maximum beat rate included. [n=11 for all groups]

* see Appendix 3

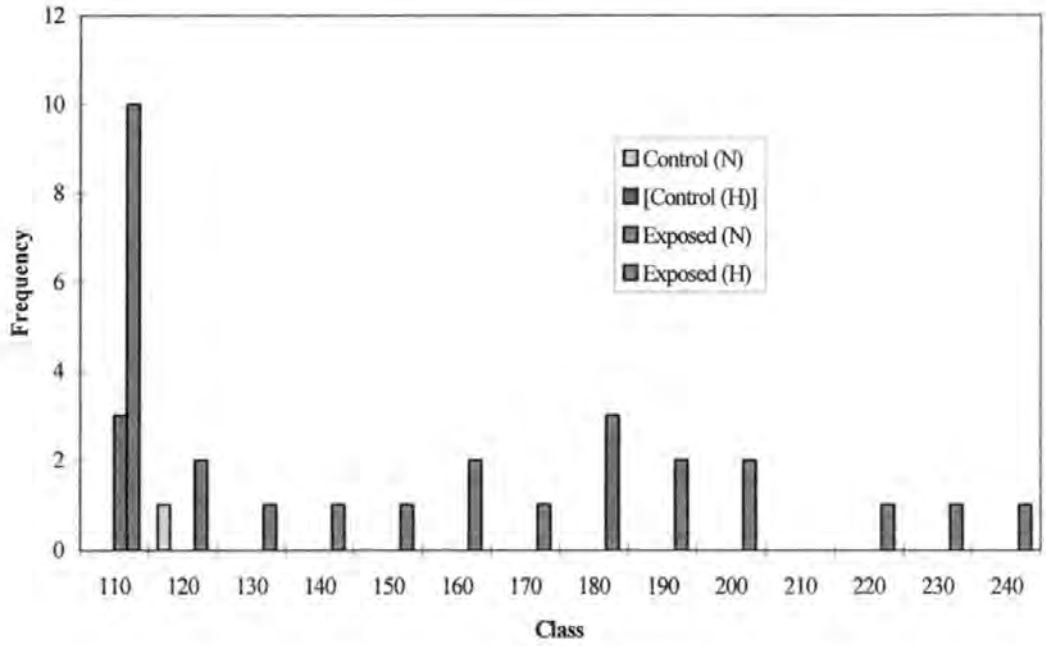


Figure 6.6b: Absolute frequencies of different scaphognathite beat rates (bpm) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions. Class boundaries given correspond to the maximum beat rate included. Note change in scale of y-axis (Frequency). [n=11 for all groups]

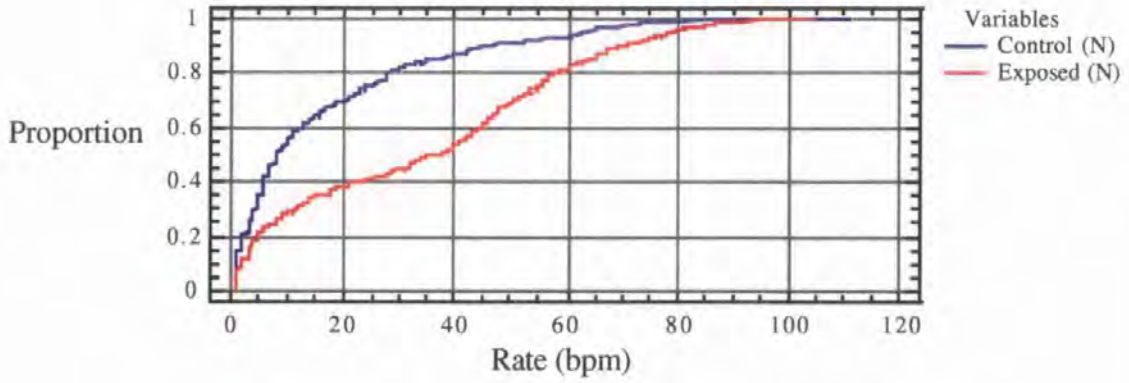


Figure 6.7: Cumulative frequency plot of the scaphognathite rates in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* in normoxic (N) conditions. [n=11 for both groups]

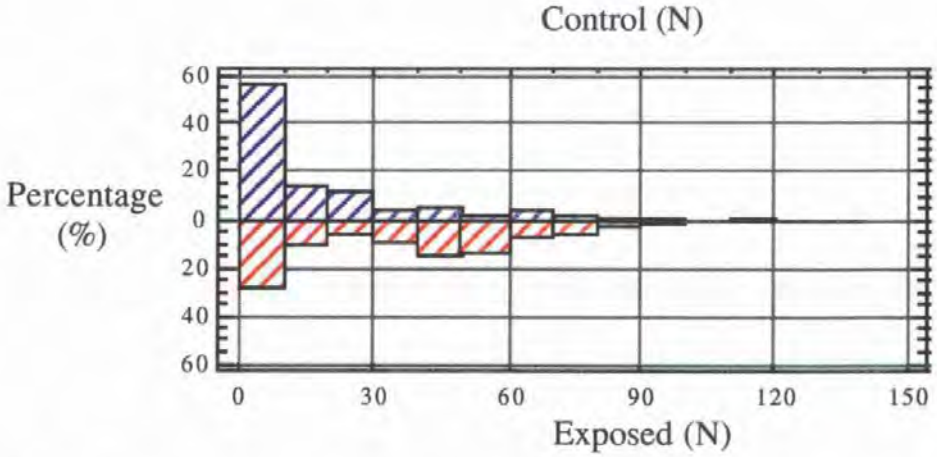


Figure 6.8: Frequency histogram of scaphognathite beat rates (beat per minute) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* in normoxic (N) conditions. [n=11 for both groups]

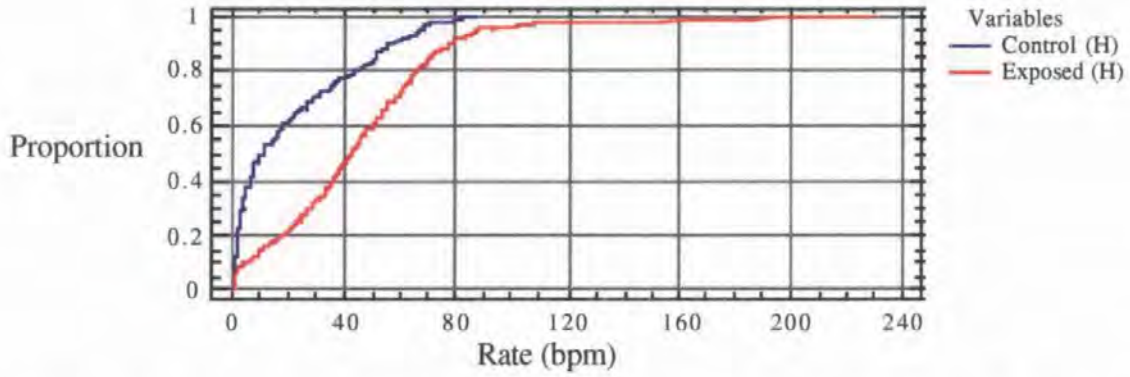


Figure 6.9: Cumulative frequency plot of the scaphognathite rate data in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* in hypoxic (H) conditions. [n=11 for both groups]

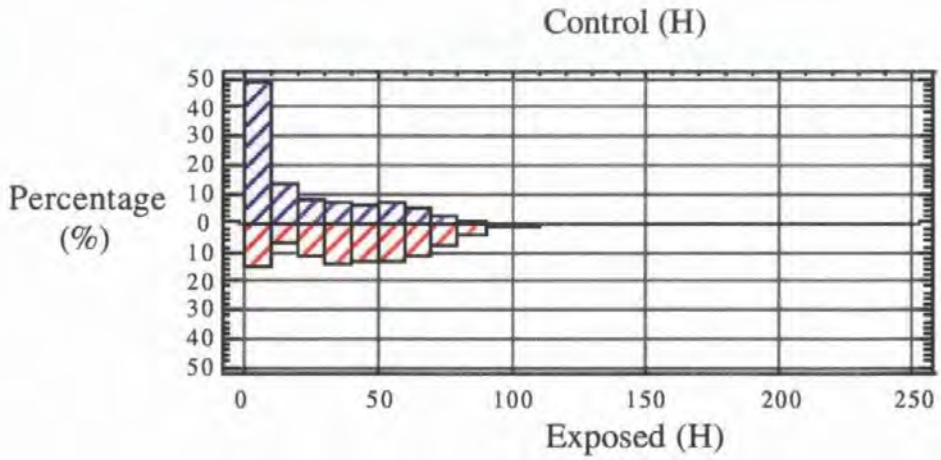


Figure 6.10: Frequency histogram of scaphognathite beat rates (beat per minute) recorded over 1 h, using only active ventilatory time (>0 bpm), in control and exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* in hypoxic (H) conditions. [n=11 for both groups]

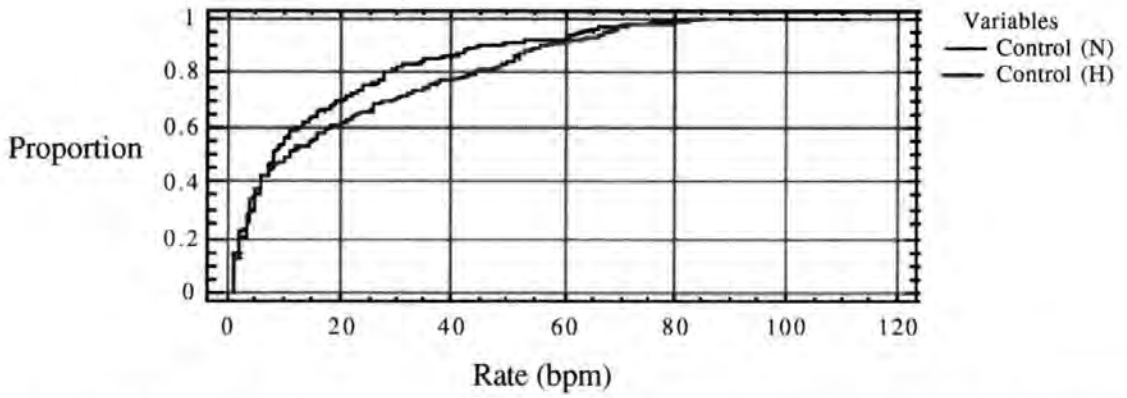


Figure 6.11: Cumulative frequency plot of the scaphognathite rate data in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in control *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions. [n=11 for both groups]

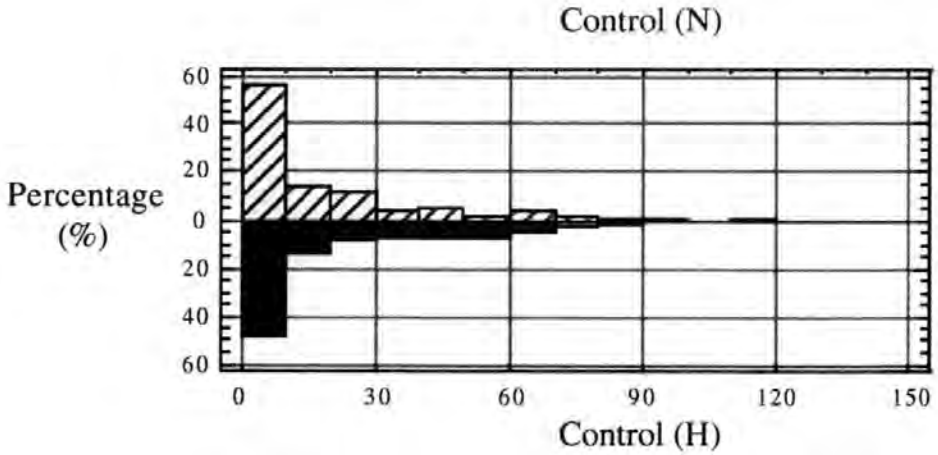


Figure 6.12: Frequency histogram of scaphognathite beat rates (beat per minute) recorded over 1 h, using only active ventilatory time (>0 bpm), in control *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions. [n=11 for both groups]

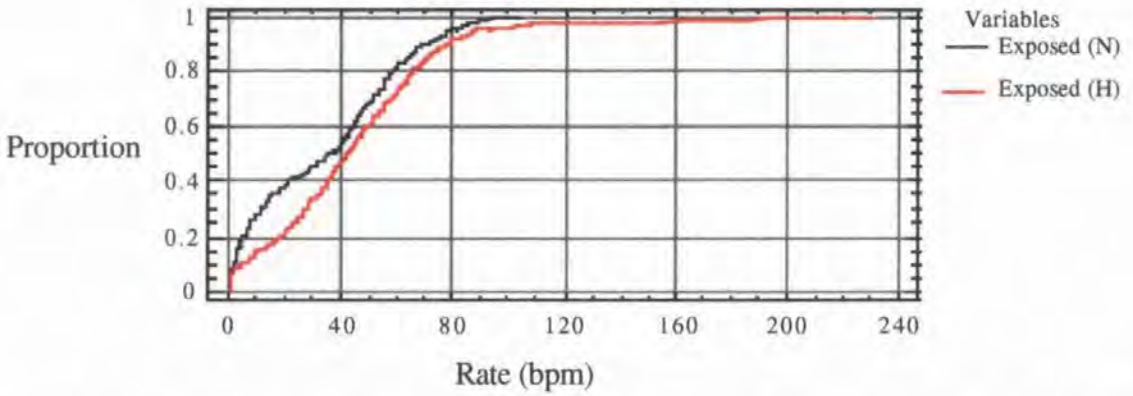


Figure 6.13: Cumulative frequency plot of the scaphognathite rate data in beats per minute (bpm) recorded over 1 h, using only active ventilatory time (>0 bpm), in exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions. [n=11 for both groups]

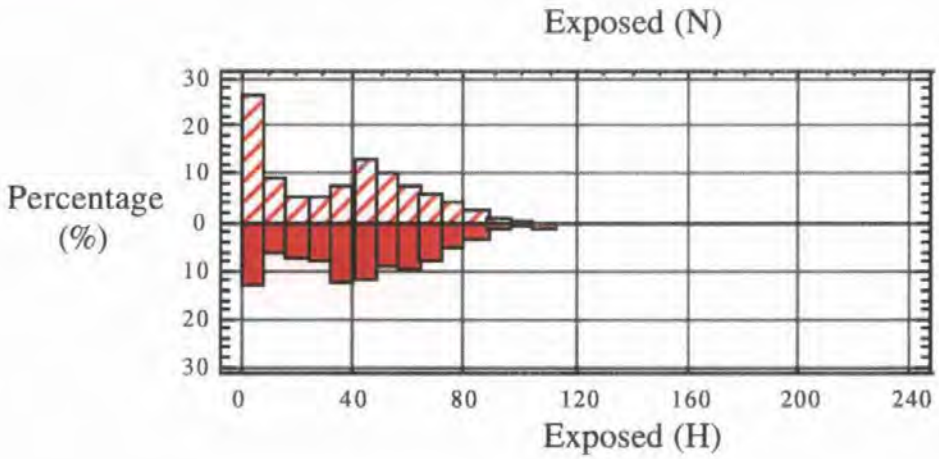


Figure 6.14: Frequency histogram of scaphognathite beat rates (beats per minute) recorded over 1 h, using only active ventilatory time (>0 bpm), in exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions. [n=11 for both groups]

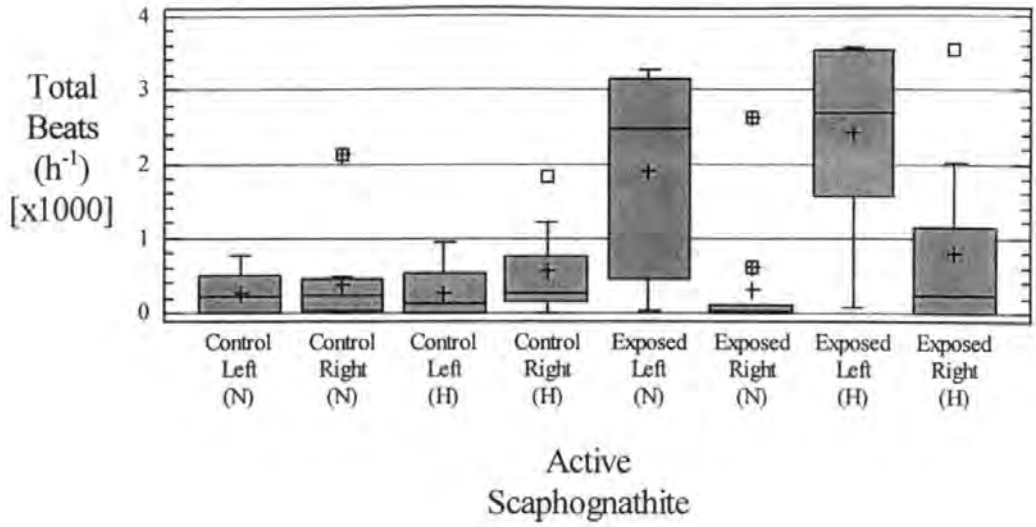


Figure 6.15: Box-Whisker plot* showing the relative contributions of left and right scaphognathites in control and exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* under normoxic (N) and hypoxic (H) conditions to the beats per hour. [n=11 for all groups]

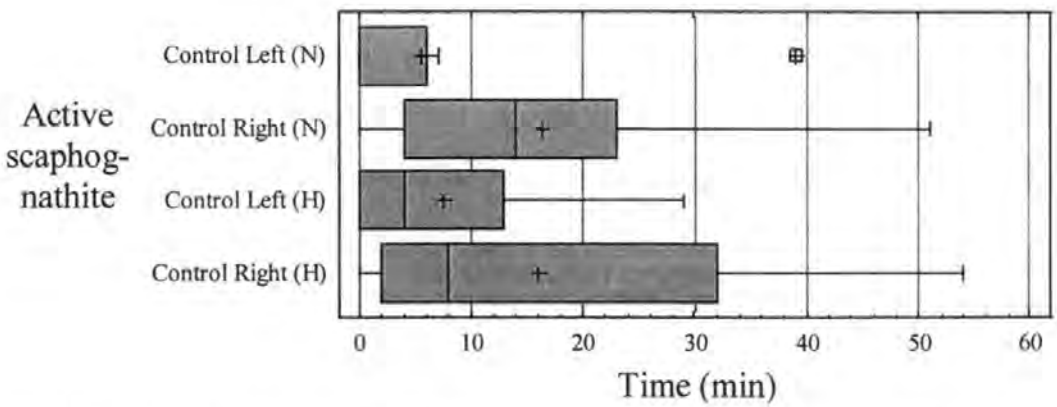


Figure 6.16: Box-Whisker plot* of time spent by control *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions with either the left or right scaphognathite active unilaterally. The same animals are used for normoxic and hypoxic measurements. [n=11 for all groups]

* see Appendix 3

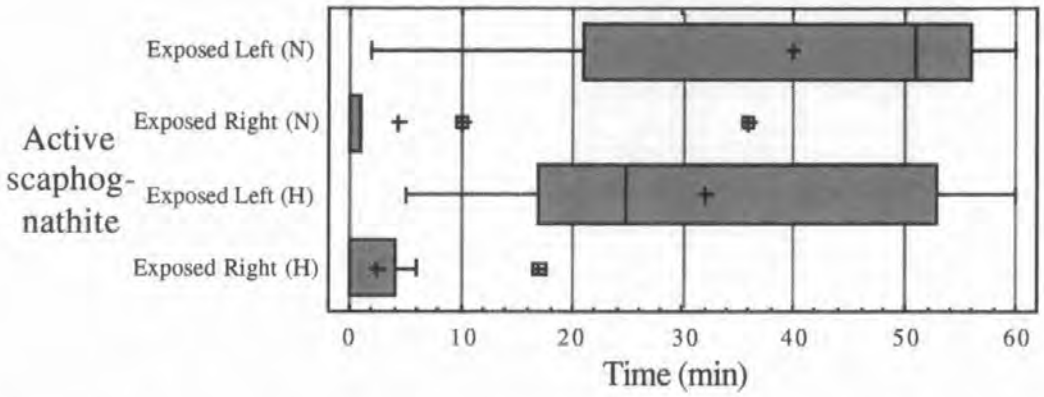


Figure 6.17: Box-Whisker plot* of time spent by exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions with either the left or right scaphognathite active unilaterally. The same animals are used for normoxic and hypoxic measurements. [n=11 for all groups]

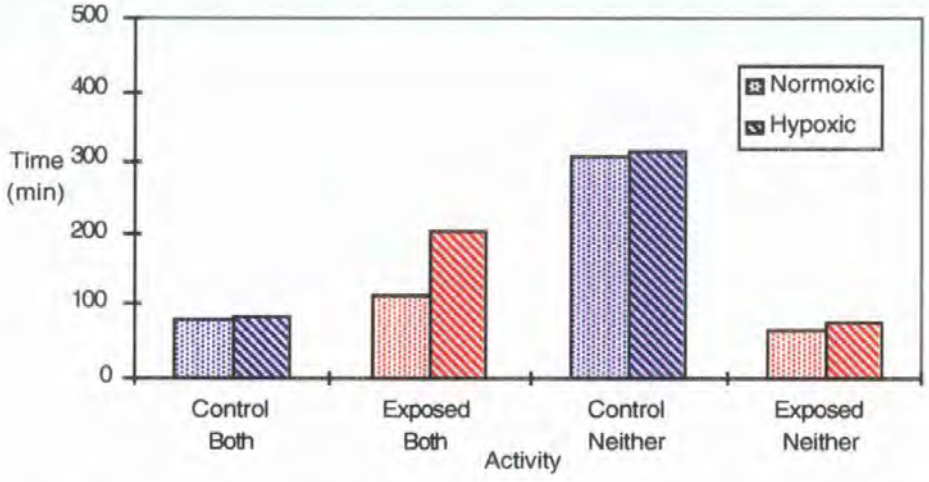


Figure 6.18: Total minutes spent by control and exposed (10 days at 500 µg Cu L^{-1}) *Carcinus maenas* in normoxic and hypoxic conditions beating with both or neither scaphognathite(s) during 1 h. [n=11 for all groups, i.e. maximum possible number of minutes: 11 x 60 min = 660 min].

* see Appendix 3

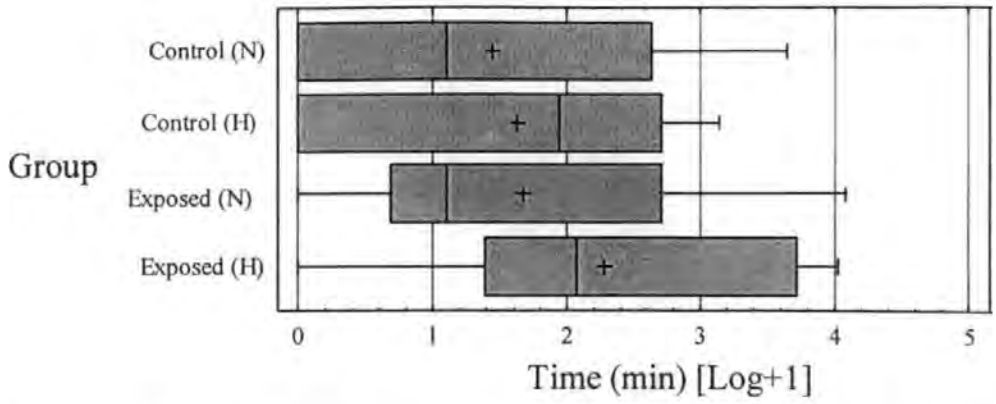


Figure 6.19: Box-Whisker plot* of time spent by control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in hypoxic (H) and normoxic (N) conditions with scaphognathites beating bilaterally. Data is [Log+1]-transformed as used for statistical comparisons. [n=11 for all groups]

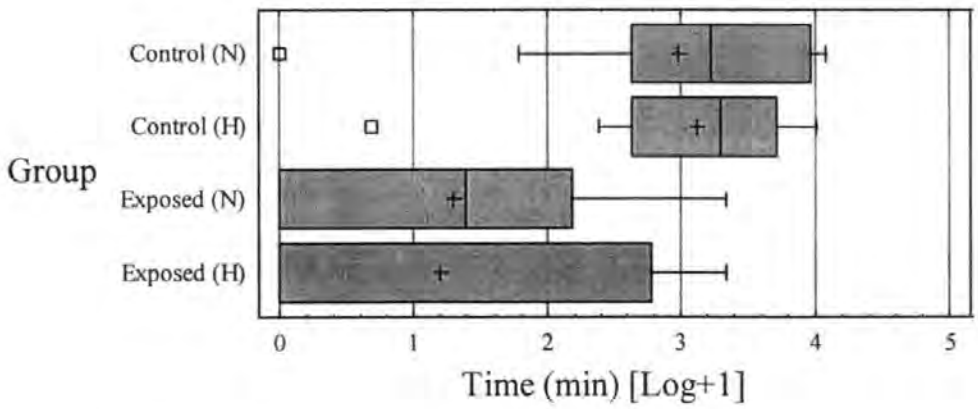


Figure 6.20: Box-Whisker plot* of time spent by control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in bilateral apnoea, in normoxic (N) and hypoxic (H) conditions. Data is [Log+1]-transformed as used for statistical comparisons. [n=11 for all groups]

* see Appendix 3

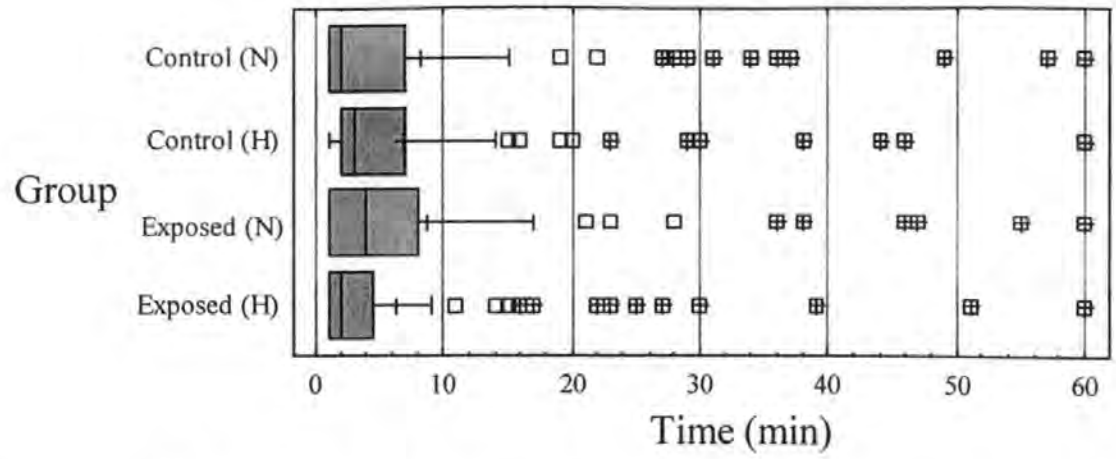


Figure 6.21: Box-Whisker plot* showing the length of ventilatory pauses (apnoea) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* under normoxic (N) and hypoxic (H) conditions. [n=11 for all groups]

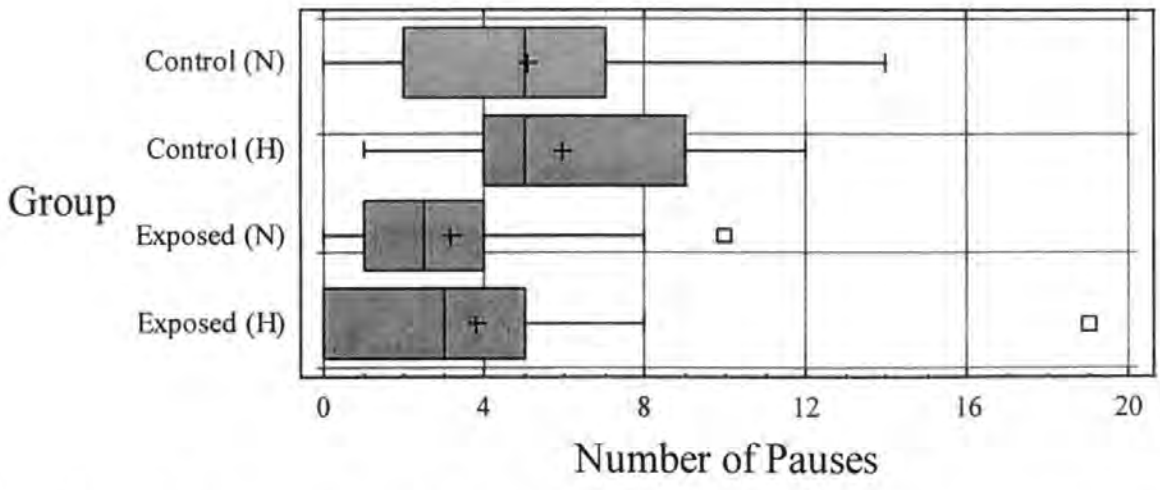


Figure 6.22: Box-Whisker plot* showing the number of unilateral ventilatory pauses (apnoea) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* under normoxic (N) and hypoxic (H) conditions. [n=11 for all groups]

* see Appendix 3

Table 6.1: Significance test results of comparisons of scaphognathite beat rates in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions, using Mann-Whitney Two-sample Comparisons (*, significant; ***, highly significant). [n=11 for all groups]

Comparison	Test Statistic (W)	p-Value
Control (N) < Exposed (N)	201233.0	<0.005***
Control (H) < Exposed (H)	247434.0	<0.005***
Control (N) < Control (H)	94182.0	0.0433*
Exposed (N) < Exposed (H)	326114.0	<0.005***

Table 6.2: Actual median and mean scaphognathite rates in beats per minute (bpm) in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions, calculated from 1 h of monitoring, using only active ventilatory time (i.e. > 0 bpm). [n=11 for all groups]

Treatment Group	Median Rate (bpm)	Mean Rate (bpm)
Control (N)	8.0	17.0
Control (H)	11.0	21.2
Exposed (N)	37.0	34.7
Exposed (H)	43.0	44.7

Table 6.3: Results of the Kolmogorov-Smirnov Two-sample Tests, comparing data distributions of ventilatory rates of control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions, including maximum distance between cumulative frequency plots (DN), the Kolmogorov-Smirnov (K-S) test statistic and significance levels (***, highly significant). [n=11 for all groups]

Comparison	DN	K-S Statistic	Significance Level
Control (N) ≠ Exposed (N)	0.38	6.15	p<0.005***
Control (H) ≠ Exposed (H)	0.41	6.88	p<0.005***
Control (N) ≠ Control (H)	0.15	2.18	p<0.005***
Exposed (N) ≠ Exposed (H)	0.17	3.36	p<0.005***

Table 6.4: Significance values for comparisons between different scaphognathite activity types in control and/or exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and/or hypoxic (H) conditions. The data are analysed using Kruskal-Wallis Two-sample Comparisons (NS, not significant [p>0.05]; *, significant; ***, highly significant). [n=11 for all groups]

Comparison	Test Statistic (Kruskal-Wallis)	p-Value
Control Left (N) ≠ Control Left (H)	1.27	0.2603 (NS)
Control Right (N) ≠ Control Right (H)	0.09	0.767 (NS)
Control Left (N) < Control Right (N)	5.96	0.0147*
Control Left (H) ≠ Control Right (H)	1.19	0.2757 (NS)
Exposed Left (N) ≠ Exposed Left (H)	0.43	0.5109 (NS)
Exposed Right (N) ≠ Exposed Right (H)	0.002	0.9666 (NS)
Exposed Left (N) > Exposed Right (N)	13.96	0.0002***
Exposed Left (H) > Exposed Right (H)	14.21	0.0002***

Table 6.5: Significance values for comparisons (One-way ANOVA) of the time spent in bilateral apnoea by control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions (NS, not significant [p>0.05]; **, very significant). [n=11 for all groups]

Comparison [Log+1] data transformations	**Test Statistic** (F-Ratio)	**p-Value**
Control (N) - Control (H)	0.11	0.7462 (NS)
Exposed (N) - Exposed (H)	0.03	0.8604 (NS)
Control (N) - Exposed (N)	11.01	0.0034**
Control (H) - Exposed (H)	13.68	0.0014**

Table 6.6: Results of Mann-Whitney Two-sample Comparisons of the number of ventilatory pauses recorded in control and exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* under normoxic (N) and hypoxic (H) conditions (NS, not significant [p>0.05]; *, significant, ***, highly significant). [n=11 for all groups]

Comparison	**Test Statistic** (W)	**p-Value**
Control (N) ≠ Control (H)	273.0	0.4709 (NS)
Exposed (N) ≠ Exposed (H)	250.0	0.8587 (NS)
Control (N) ≠ Exposed (N)	159.5	0.5263 (NS)
Control (N) ≠ Exposed (H)	174.5	0.1129 (NS)
Control (H) > Exposed (N)	129.5	0.0041***
Control (H) > Exposed (H)	143.0	0.01*

Table 6.7: Summary of scaphognathite activity patterns in control and copper-exposed (10 days at 500 μg Cu L^{-1}) *Carcinus maenas* in normoxic (N) and hypoxic (H) conditions (NS, not significant [p>0.05]; *, significant [p<0.05]; ***, highly significant [p<0.005]). [n=11 for all groups]

Scaphognathite activity	
Total Scaphognathite Beats‡	**Ventilatory Rate**
Control (N) < Control (H)***	Control (N) < Control (H)*
Exposed (N) < Exposed (H)***	Exposed (N) < Exposed (H)***
Control (N) < Exposed (N)***	Control (N) < Exposed (N)***
Control (H) < Exposed (H)***	Control (H) < Exposed (H)***
Bilateral Apnoea	**Unilateral Apnoea (Frequency)**
Control (N) ≠ Control (H) (NS)	Control (N) ≠ Control (H) (NS)
Exposed (N) ≠ Exposed (H) (NS)	Exposed (N) ≠ Exposed (H) (NS)
Control (N) > Exposed (N)***	Control (N) ≠ Exposed (N) (NS)
Control (H) > Exposed (H)***	Control (H) > Exposed (H)*
Unilateral Apnoea (Duration)	**Total Minutes in Apnoea**
Control (N) ≠ Control (H) (NS)	Control (N) > Control (H)†
Exposed (N) ≠ Exposed (H) (NS)	Exposed (N) > Exposed (H)†
Control (N) ≠ Exposed (N) (NS)	Control (N) > Exposed (N)†
Control (H) ≠ Exposed (H) (NS)	Control (H) > Exposed (H)†
Bilateral Activity	**Total Beats Left/Right**
Control (N) ≠ Control (H) (NS)	Control (L/N) ≠ Control (R/N) (NS)
Exposed (N) ≠ Exposed (H) (NS)	Control (L/H) ≠ Control (R/H) (NS)
Control (N) ≠ Exposed (N) (NS)	Exposed (L/N) > Exposed (R/N)***
Control (H) ≠ Exposed (H) (NS)	Exposed (L/H) > Exposed (R/H)***
Time in Unilateral Activity	
Control (L/N) < Control (R/N)*	Exposed (L/N) > Exposed (R/N)***
Control (L/H) ≠ Control (R/H) (NS)	Exposed (L/H) > Exposed (R/H)***
Control (L/N) ≠ Control (L/H) (NS)	Exposed (L/N) ≠ Exposed (R/N) (NS)
Control (R/N) ≠ Control (R/H) (NS)	Exposed (L/H) ≠ Exposed (R/H) (NS)

‡significance values based on result of Advanced Regression/General Linear Models, i.e. significance of factors copper and hypoxia in general; †observations made without significance tests.

CHAPTER 7

GILL STRUCTURE AND METALLOTHIONEIN CONCENTRATIONS IN *CARCINUS MAENAS* FROM TWO ESTUARIES WITH DIFFERENT LEVELS OF TRACE METALS

Abstract

The gill structure of *Carcinus maenas* (L.) taken directly from a relatively clean site (Avon Estuary, Devon), a metal-polluted site (Restronguet Creek, Cornwall), and of animals transplanted from the clean site to the polluted site for 8 days, was compared to test the hypothesis that gill ultrastructure is affected by exposure to trace metals in the field. In addition, metallothionein concentrations and total tissue copper concentrations were measured in the gills and midgut gland of crabs from each group.

Gill and midgut gland tissue of crabs from Restronguet Creek showed significantly higher total copper and/or metallothionein concentrations than corresponding tissues of crabs from the Avon Estuary. Tissues of transplanted animals gave intermediate values of metallothionein which were not significantly different from crabs from either field site.

Gill ultrastructure of crabs from the two field sites showed inter-population differences. In the posterior gills of crabs from Restronguet Creek, striated cells showed a significantly higher density of basal plasmalemma folds than in the corresponding tissues from Avon Estuary crabs. In the pillar cells, the density of apical membrane folds was significantly higher in posterior gills of Avon Estuary than Restronguet Creek crabs. The gill epithelia of transplanted crabs showed differences from the gill tissue of each field site. Ultrastructural changes in the gill epithelial pillar cells of the transplanted crabs were similar to those reported for experimental metal exposures, including increased apical vacuolation (posterior gills) and distended mitochondria (anterior gills). In all groups, anterior gill tissue had a thinner cuticle and fewer nephrocytes than the posterior gills. The algal/bacterial surface layers were thickest in crabs from Restronguet Creek and had a different composition and structure from other groups.

The morphological differences between the gills (especially the osmoregulatory tissue) of *Carcinus maenas* from Restronguet Creek and the Avon Estuary indicate that the gills may be functionally different. Changes in the ultrastructure of gills of transplanted crabs suggests a degree of morphological adaptation in Restronguet Creek crab gills in response to their (trace metal contaminated) environment.

7.1 Introduction

Although total tissue metal burdens can reflect the history of metal exposure of an animal in its environment, differences in physiological state and trace metal uptake mechanisms of the organism have to be considered (Bryan, 1967, 1968; Depledge, 1989b; Depledge and Rainbow, 1990; Bjerregaard, 1991; Pedersen and Lundebye, 1996; Rainbow, 1997a). Depending on the mode of metal uptake (i.e. by ingestion with food; direct uptake from the water column), and the resulting metal transfer and detoxification mechanisms, metals will be stored preferentially in certain tissues. In Restronguet Creek, *Carcinus maenas* is exposed to elevated levels of trace metals (in particular copper) in the water and the sediment (Bryan and Gibbs, 1983; Somerfield *et al.*, 1994; Hubert, 1995; Langston *et al.*, 1995). It can be assumed that any foraging animal, such as *C. maenas*, is exposed to higher levels of bioavailable copper than measured in the water column, as it comes into contact with interstitial water of the highly contaminated sediment. Considerable concentrations of copper are, however, bioavailable directly from the water column in Restronguet Creek (Hubert, 1995). Although copper is an essential metal in the physiology of this species (e.g. as a constituent of the oxygen-binding haemolymph protein, haemocyanin), it becomes overtly toxic if levels exceed the animal's regulatory capabilities (Hebel *et al.*, 1997).

In crustaceans, the first site of uptake of water-borne trace metals is the gills (see Section 1.3.1 for a summary of gill structure and function). The effects of trace metals on crustacean gill ultrastructure, therefore, have been documented in many aquatic decapods, including the prawn *Crangon crangon* (Papathanassiou, 1985), and the crabs *Scylla serrata* (Krishnaja *et al.*, 1987) and *C. maenas* (Nonnotte *et al.*, 1993; Lawson *et al.*, 1995) (see also Table 2.2). The majority of these studies, however, were based upon responses to laboratory exposures of a single trace metal and relied on qualitative observations rather than quantitative morphometric techniques. While the former approach may be appropriate when dealing with acute effects of exposure, morphometric analyses identify more subtle tissue changes and differences, and are especially useful in comparative studies (e.g. Hughes and Perry, 1979; Papathanassiou and King, 1983; Sunila, 1986). In addition, there is a dearth of studies involving trace metal exposure in the field, where animals are generally exposed to a "cocktail" of different trace metals, including copper, zinc and cadmium, rather than a single trace metal in solution.

This study combines qualitative and quantitative ultrastructural analyses of the gills of *C. maenas* taken directly from the field, to assess the effects of chronic (and acute) trace metal exposure on gill ultrastructure.

7.2 Materials and Methods

7.2.1 Collection

Carcinus maenas were collected from two estuaries with different levels of trace metal contamination. In March 1995 and November 1996, crabs were taken from the Avon Estuary (Devon) near the village of Bantham (Figs 7.1a to c; 7.2). This estuary was chosen as a "control" site as water metal loads are low [Environment Agency (EA), pers. com.]. Levels of dissolved copper at the EA sampling site Hatch (Fig. 7.1c) were below 2.5 μg L^{-1} (throughout 1996), and total zinc levels in the water samples rarely exceeded 9 μg L^{-1} throughout the year (Environment Agency, pers. com.). In addition, earlier work (see Section 1.3.1 and previous chapters) established that the gill ultrastructure of animals collected from this estuary corresponded with that reported previously as typical of the gill structure of healthy *C. maenas* (e.g. Goodman and Cavey, 1990). Restronguet Creek (Fal Estuary, Cornwall) (Figs 7.1d, 7.3a) was chosen as a source of "polluted" animals. The site, with a long history of mining and high metal loads in the water and sediment (Somerfield *et al.*, 1994), is used widely to study the chronic effects of trace metals in the environment (eg. Somerfield *et al.*, 1994; Perryman, 1997). Although mining has ceased, mine flooding and resuspension of sediment-bound metal causes increased levels of metals in the water. Copper levels are especially elevated. A recent study (Hubert, 1995) reported 78.4 μg L^{-1} total copper concentration in the water at the mouth of Penpol Creek; taking into account metal speciation, the amount of bioavailable copper reaches 29.4 μg L^{-1} (Hubert, 1995). In March 1995 and June 1996, *C. maenas* were collected from the convergence of Penpol and Restronguet Creeks (Fig. 7.1d). At each sampling site, crabs were collected using drop nets (baited with various species of fish) (Fig. 2.2). Only males with green carapaces and of similar size (*c.* 6 cm carapace width) were collected. The crabs were transported to the laboratory in Plymouth in buckets of local seawater (salinity of 35) and dissected immediately for the analysis of gill ultrastructure, total copper concentration and metallothionein concentrations.

In addition to these collections, a transplant experiment was carried out in October 1996. Eight male crabs (sizes as reported earlier) were collected at Bantham and transported to Restronguet Creek in buckets of local seawater (salinity of 35). A cage (80

cm x 50 cm x 25 cm), constructed of stainless steal mesh with 8 individual compartments (25 cm x 20 cm x 25 cm), was positioned in the same area where previous crab samples had been collected (Fig. 7.3b). The cage was held in place securely by long pegs (30 cm) protruding from each of the four corners and inserted into the sediment. Crabs were placed individually in the 8 chambers. Escape of the experimental animals was avoided by attaching a "floor" of netting to the open base of the cage, partially burying the mesh walls in the sediment and by securely fastening the perspex lid of each individual chamber. The cage was covered partially with seaweed and rocks to provide shelter for the crabs. Seven animals were recovered successfully after 8 days, and transported immediately to Plymouth (in buckets of seawater) for dissection.

7.2.2 Atomic Absorption Spectroscopy

Tissue copper levels were measured in crabs collected in March 1995 from the Avon Estuary and Restronguet Creek). Gill and midgut gland tissue was dissected from the crabs (n=5 for each collection site) and freeze dried. The tissues were digested for copper determination in a two-step process, entailing a 12 h acid digestion, followed by microwave digestion. Tissue copper content was determined using a Spectra AA-400 Plus Flame Atomic Absorption Spectrometer (AAS) (Varian) and statistical differences determined by One-way ANOVA (*Statgraphics Plus 2.1 for Windows).* Total copper of gill tissue was not determined for transplanted animals due to insufficient tissue available to carry out both AAS and metallothionein analyses.

7.2.3 Metallothionein

Metallothionein (MT) concentrations were measured in crabs collected directly from the two field sites [June (Restronguet Creek, n=8) and November (Avon Estuary, n=15) 1996] and from crabs after 8 days in the transplantation cage [October 1996, n=7]. Gill and midgut gland tissue were dissected and snap frozen in liquid nitrogen. Metallothionein quantification was carried out as described in Section 4.2.6. Statistical comparisons of MT concentrations were carried out by One-Way ANOVA (*Statgraphics Plus 2.1 for Windows*).

7.2.4 Gill ultrastructural processing and assessment

Gill tissue from crabs sampled directly from the two field sites [Avon Estuary (March 1995 and November 1996) and Restronguet Creek (March 1995 and June 1996)] and from the transplantation cage (October 1996) was removed for transmission electron microscopy (TEM) by cutting at the base of an osmoregulatory (posterior, number 8) and a respiratory (anterior, number 5) gill (Compère *et al.*, 1989). Further processing for analysis

by TEM was carried out as described in Section 2.2.2. In addition to looking at gill ultrastructure, various morphometric analyses were carried out on the gills of each crab group.

As haemocyte and nephrocyte numbers were found to be affected by metal exposure in the laboratory (see Section 2.3), their numbers were determined using TEM by counting the cells present in a 300 μm-portion of each lamella [equivalent to the length of 3 grid squares of the copper grids carrying the thin tissue sections (see Section 2.2.2)].

Structural characteristics of the striated cells (posterior gill), chief cells (anterior gill) and pillar cells (posterior and anterior gills) were quantified by counting the number of apical (pillar and chief cells) or basal membrane (striated cells) invaginations, and by determining the relative mitochondrial area volumes. Apical and basal membrane invaginations were counted using TEM micrograph negatives, extrapolating counts to 1 μm of cuticle length. Relative mitochondrial area volumes within epithelial cells were determined by overlaying the micrograph negatives with an array of points (Glagoleff, 1933; Williams, 1977) and determining the mitochondrial area volume ($V_{V/mit}$) [i.e. the ratio (given as a percentage value) of the actual number of points coinciding with mitochondria on the negative over the number of possible hits (i.e. the total number of points applied to the micrograph)]. For this determination, micrographs were taken at a magnification of x 6000, except for the $V_{V/mit}$ of chief cells which was determined at a magnification of x 12000. The area of the points applied was measured against the background using a colour video camera (DXC-151P, Sony) in conjunction with an image analyser system (Quantimet 570, Cambridge Scientific Instruments) to determine the area fraction of the points. The calculated ratio confirmed that the size of the points applied to the micrographs did not affect the accuracy of counts. To ensure that the point array applied to the micrographs allowed sufficient precision in estimating the $V_{V/mit}$, the relative standard error (RSE) (Hally, 1964) was calculated following a pilot experiment, using the formula:

$$RSE = \frac{\sqrt{1 - V_{V/mit}}}{\sqrt{n}}$$

where n=number of points to be applied. This method ensured that the standard error due to sample number calculated remained at approximately 5% (although overall standard error may be higher due to within sample variability).

Cuticle thickness was measured on micrograph negatives of gill sections of crabs from each group, taking three replicate measurements per animal at random along the tissue section. The thickness of the algal/bacterial layer was determined by measuring the mean width of the total cuticular layer (i.e. cuticle and algal/bacterial layer) by image analysis, taking three replicate measurements of randomly chosen tissue portions along the methylene-blue section, and subtracting the previously determined mean measurement of cuticle width from this.

Statistical comparisons of results from these morphometric assessments were carried out using Advanced Regression/General Linear Models (GLM) or One-way ANOVA (*Statgraphics Plus 2.1 for Windows).*

7.3 Results

7.3.1 Tissue copper levels

Gill and midgut gland (mgg) tissue of *C. maenas* collected at Restronguet Creek contained significantly higher levels of copper than corresponding tissue from crabs collected from the Avon Estuary (Fig. 7.4) (One-way ANOVA; gill: $F=18.09$, $p=0.0028$; mgg: $F=22.29$, $p=0.0015$). Tissue copper levels (gill versus mgg) were not significantly different for Avon Estuary crabs ($F=0.83$; $p=0.3878$), however, the mgg contained significantly higher copper concentrations than the gills of crabs from Restronguet Creek ($F=16.76$; $p=0.0035$) (One-way ANOVA).

7.3.2 Tissue metallothionein concentrations

Metallothionein concentrations in the gill and midgut gland (mgg) of crabs from the two field sites and from the transplanted animals are shown in Figure 7.5. Gill MT concentrations of Restronguet Creek crabs were significantly higher than those of Avon Estuary crabs (One-way ANOVA; $F=4.66$; $p=0.0182$). The MT concentrations in the gill tissue of transplanted animals were intermediate and not significantly different from the other two groups. Metallothionein measurements in the mgg of Restronguet Creek crabs were significantly higher than values in crabs from the Avon Estuary and the cage (One-way ANOVA; $F=8.75$; $p=0.003$). All MT mgg measurements were significantly higher than equivalent gill tissue samples [One-way ANOVA; F-Ratios = 283.15 (Avon), 103.31 (Cage), 412.71 (Restronguet); $p<0.00001$].

7.3.3 Haemocytes

There was no obvious difference in the ultrastructure of haemocyte cells between Restronguet Creek, Avon Estuary and caged crabs. Haemocytes were of irregular shape

and contained mostly spherical or ovoid granules of different sizes (up to 1 μm in diameter) which had either a uniformly electron dense or a "honeycombed" appearance (Fig. 7.6). Some of these granules represent lysosomes. The nucleus was either central or displaced to one side (Fig. 7.6). Endoplasmic reticula and small mitochondria were present in the cytoplasm between granules and the nucleus. Mean total haemocyte numbers in the anterior gills of Avon Estuary and caged crabs were not significantly different (One-way ANOVA; $F=1.1$; $p=0.3088$). Haemocyte numbers in the posterior gills of the three crab groups were not significantly different (One-way ANOVA; $F=1.53$; $p=0.2312$) (Fig. 7.7). There were no significant differences in haemocyte counts between the anterior and posterior gills of caged crabs ($F=0.19$; $p=0.6736$) and Avon Estuary crabs ($F=0.02$; $p=0.8784$) (One-way ANOVA).

7.3.4 Nephrocytes

Nephrocytes, generally present in association with the intralamellar septum, were distributed throughout the lamellar section either in clusters or singularly. Pedicels were present on the cellular margins, with a central nucleus being visible (if it coincided with sectioning plane), as well as a large central vacuole surrounded by smaller satellite vacuoles within the cytoplasm (Fig. 7.8). There was considerable variation in size (depending on sectioning plane) and internal appearance, the vacuoles containing different amounts and forms of electron dense material. Mean nephrocyte numbers (Fig. 7.9) were not significantly different in the anterior gill samples of Avon Estuary and caged crabs (One-way ANOVA; $F=0.91$; $p=0.355$). The posterior gills of crabs from each of the three groups did not differ significantly (One-way ANOVA; $F=0.88$; $p=0.4244$) (Fig. 7.9). For Avon Estuary and cage samples, the posterior gills contained significantly higher mean nephrocyte numbers than the anterior equivalent in both cases (One-way ANOVA; Avon: $F=25.98$, $p<0.0000$; Cage: $F=5.31$, $p=0.0467$).

7.3.5 Striated cells

Striated cells were the predominant epithelial cell type in the posterior gill samples, reaching up to 15 μm into the lamellar haemolymph space. Qualitative TEM analysis found no differences between the typical features of striated cells in gills from the three sample groups (Fig. 7.10a). Both apical and basal cell membranes (Figs 7.10b and c) were highly infolded, the latter reaching deep (approximately 1/2 to 2/3 of the total cell depth) into the epithelial cell layer. Small vacuoles were present amongst the apical folds in striated cells from each sample group. Mitochondria were present throughout the cell (although not amongst the apical plasmalemma), with elongated mitochondria lying within

the basal membrane folds (Fig. 7.10c). The nucleus was located in the upper half of the cell (i.e. closer to the cuticle surface than the basal lamina) but its obvious presence depended on the sectioning plane chosen. Generally, morphometric analysis of striated cells confirmed the uniformity of this cell type in all crab groups, although the mean number of basal membrane invaginations (Fig. 7.11) in crabs from Restronguet Creek was significantly higher than in the Avon Estuary crabs (Advanced regression/GLM; F=3.51; p=0.0437). Striated cells from caged animals had an intermediate mean number of basal membrane infoldings. The number of apical membrane invaginations of striated cells in the posterior gills did not differ significantly in any crab type (Advanced regression/GLM; F=1.64; p=0.2113) (Fig. 7.12). In addition, mitochondrial area volumes showed no significant differences between the three crab groups (Advanced regression/GLM; F=0.32; p=0.727) (Fig. 7.13).

7.3.6 Chief cells

Chief cells were the predominant cell type in the epithelia of the anterior gill tissue. Their appearance did not vary between crabs from the Avon Estuary and the cage. Chief cells were approximately 2-5 μm in height and contained many mitochondria (Fig. 7.14). Vacuoles, of various sizes and shapes, were present within these epithelial cells in both sample groups. Apical plasmalemma, however, were less elaborately folded than in the striated cell type. Some degree of basal membrane folding was present (although not in all cases), without direct association of mitochondria as seen in striated cells. Analysis of apical membrane invaginations (Fig. 7.15) and mitochondrial area volumes (Fig. 7.16) showed there were no significant differences between anterior gill tissue from Avon Estuary and transplanted (cage) animals (Advanced regression/GLM; apical membranes: F=1.39, p=0.2565; mitochondrial area volumes: F=1.56, p=0.2312).

7.3.7 Pillar cells

Pillar cells were present in both the anterior and posterior gill lamellae. They were identified by the presence of electron dense microtubular bundles either spanning from one cuticle to the other across the haemolymph space, or being visible in part in the base of the cell (due to sectioning plane) (Fig. 7.17a). Cells were either multinucleate or contained a single large nucleus. Mitochondria were present within the cell, although their relative area depended upon the spread of the microtubular fibres which lead to an apparent reduction in the number of mitochondria present. Apical membrane infoldings were present and were attached to the endocuticle in all pillar cells. The number of apical membrane infoldings were not significantly different (Advanced regression/GLM; F=0.53; p=0.481)

in pillar cells of the anterior gills of crabs from the Avon Estuary and caged animals; however, the posterior gills of Avon Estuary crabs had significantly higher apical membrane counts than either caged or Restronguet Creek crabs (Advanced regression/GLM; F=5.22; p=0.0135) (Fig. 7.18). The reduction in apical membrane infoldings in the posterior gills of caged crabs coincided with larger vacuoles being present in association with the membrane (Fig. 7.17b). Apical membrane counts in anterior gills of individuals from the Avon Estuary were significantly lower than in posterior gills of crabs from the same site (Advanced regression/GLM; F=5.14; p=0.0359). Membrane counts in the anterior and posterior gills of caged animals were not significantly different (Advanced regression/GLM; F<0.00; p=0.9597).

Mitochondrial area volumes ($V_{V/mit}$) in pillar cells (Fig. 7.19) from the anterior gills were significantly lower in Avon Estuary than caged crabs (Advanced regression/GLM; F=5.97; p=0.0284); the latter contained distended mitochondria (Fig. 7.17c). There were no significant differences in mitochondrial area volume between posterior gills of the three crab groups (Advanced regression/GLM; F=1.31; p=0.29). In crabs from the Avon Estuary, the mitochondrial area volume was significantly higher in the pillar cells of the posterior than the anterior gills (Advanced regression/GLM; F=35.16; p<0.0000). In caged crabs, mitochondrial area volumes of pillar cells in the anterior and posterior gills were not significantly different (Advanced regression/GLM; F=0.53; p=0.4914).

7.3.8 Cuticle width and algal/bacterial layers

Generally, the cuticle of the anterior gill was thinner than that of the posterior gill in crabs of the same group (Fig. 7.20). This difference in cuticle width was significant for caged crabs (F=6.79; p=0.0313) but not for Avon Estuary crabs (F=0.1; p=0.7535) (Advanced regression/GLM). There were no significant differences between cuticle widths of anterior gills of Avon Estuary and caged crabs (F=0.17; p=0.686), and posterior gills of the three crab groups (F=1.59; p=0.2275) (Advanced regression/GLM).

The total cuticular layer width (i.e. the sum of cuticle thickness and algal/bacterial layer) of the anterior gill lamellae was significantly smaller in crabs taken directly from the Avon Estuary compared with transplanted individuals (Advanced regression/GLM; F=5.92; p=0.0301) (Fig. 7.20). The cuticular layer width of the posterior gills of Restronguet Creek crabs was significantly greater than that of Avon Estuary crabs (Advanced regression/GLM; F=8.23; p=0.0018). Transplanted animals showed intermediate values.

Subtraction of the mean cuticle width values from mean cuticular layer measurements showed differences in the amount of algal/bacterial layers (Fig. 7.20). These measurements corresponded with differences in the structure of the algal/bacterial layers on the cuticle surfaces, which, although showing considerable variation along the lamella (and between gills from the same group), were sufficient to affect the mean thickness of the total cuticular layer determined by image analysis (Fig. 7.21). The most common appearance of the cuticle surface of the anterior gills of Avon Estuary crabs was a thin (50 nm) layer of darkly-stained detrital matter adhering to the surface, with only occasional algal/bacterial cells along the lamella. Posterior gills of these crabs often had a uniform monolayer of algae/bacteria, adhering closely to the cuticle surface. Often, posterior gills of crabs from Restronguet Creek had a multi-layered algal layer, on occasions exceeding the width of the cuticle by a factor of x 2. Algal/bacterial layers on the gill surfaces of transplanted animals were diverse, with apparently no dominant layer type. At dissection, the gills in both gill chambers of transplanted animals were partially contaminated with fine, dark particles. In contrast, gills of animals from the two field sites appeared "clean", as shown in Figure 1.3 (Chapter 1).

7.4 Discussion

Elevated copper levels in the gill and midgut gland of animals collected at Restronguet Creek compared with crabs from the Avon Estuary are consistent with the different concentrations of the metal in the surrounding water and sediment of the two field sites (Somerfield *et al.*, 1994; Hubert, 1995; Environment Agency, pers. com.). In each group of crabs, the midgut gland contained higher levels of copper than the gills, highlighting the function of the midgut gland of crustaceans as a storage organ for metals [e.g. in the form of granules which consequently can be egested with the faeces (Hopkin and Nott, 1980)]. These metal-rich granules are able to reduce the pool of soluble copper in the tissue considerably (Pedersen and Lundebye, 1996). The role of the midgut gland as an accumulation site for metals is illustrated by the high concentrations of metal-binding protein, metallothionein (MT), present in the tissue. Gills and midgut glands of crabs from Restronguet Creek contained significantly higher concentrations of MT than the corresponding tissues of crabs from the Avon Estuary.

The copper concentrations in the midgut gland of crabs from Restronguet Creek exceeded those of animals from the Avon Estuary by a factor of *c.* x 6, however, the difference in MT was only a factor of x 1.5. These measurements indicate that tissue

copper concentrations are not directly proportional to the concentration of metallothionein in the tissue. A similar observation was made by Pedersen and Lundebye (1996) for *C. maenas* from these two sites, who suggested that this difference may reflect an increase in the turn-over rate of MT, allowing metals to be passed on for storage as part of granules more rapidly. Metallothionein plays an important role in the transport and distribution of essential metals (especially copper and zinc) within the organism, but also serves as a detoxification mechanisms for non-essential metals (e.g. cadmium) and excess amounts of essential metals (Engel and Brouwer, 1984). Being part of the digestive system, the midgut gland of crustaceans receives metals ingested with the food, as well as metals transported away from other tissues, and is generally involved in the detoxification of trace metals and in maintaining these at homeostatic levels (Bryan, 1968; Rainbow and Scott, 1979; Viarengo and Nott, 1993). The gills of *C. maenas* lack such a function and, consequently, contain a lower homeostatic MT concentration than midgut gland.

Transplanting Avon Estuary crabs to the chronically polluted site at Restronguet Creek provided an insight into the effect of a pollution incident in the field on animals adapted to clean conditions (i.e. acute exposure). Metallothionein concentrations in gill and midgut gland tissue from transplanted crabs were elevated compared with crabs taken directly from the Avon Estuary, although the difference was not significant. Increase in MT concentrations results from a cellular response to elevated environmental metal concentrations (Viarengo and Nott, 1993). A longer exposure period could be applied to test if MT concentrations in the transplanted animals would reach the same concentrations as present in the tissues of the Restronguet Creek population. Recent physiological testing of *C. maenas* from the Fal Estuary indicated that Restronguet Creek animals had a reduced ability to respond to an added stress ("challenge") in the laboratory (Bamber and Depledge, in press) and implicated the osmoregulatory gill epithelia as possible targets for the effects of chronic metal contamination in the field. These physiological measurements, and increases in metallothionein concentrations, therefore, warrant analysis of gill tissue at TEM level to assess if changes in metal concentration, metallothionein concentrations and physiological performance manifest themselves in ultrastructural gill changes.

Haemocytes have been shown previously to be affected by trace metal exposure (Bubel, 1976; Lawson *et al.*, 1995). Crustacean haemocytes have a number of different functions, including lipoprotein and carbohydrate transport (e.g. Sewell, 1955; Johnston *et al.*, 1973), and a cellular defence mechanism against bacteria present in the haemolymph

(Smith and Ratcliffe, 1980). Haemocytes have been linked to a detoxifying function as increased numbers were observed in *C. maenas* gill tissue following laboratory exposure to sublethal levels of copper (Lawson *et al.*, 1995; see Sections 2.3; 4.3.1), i.e. as a detoxification response to acute exposure. Analysis of both anterior and posterior gill tissue in crabs from the Avon Estuary, Restronguet Creek and the transplant cage, however, revealed no significant differences in the number of haemocytes present. Exposure to a range of water-borne metals in the field, therefore, does not support a detoxification function attributed to the haemocytes. Indeed, Rtal and Truchot (1996) showed recently that exposure of *C. maenas* to copper resulted in insignificant amounts of exogenous metal being associated with the cellular fraction of the haemolymph. The exogenous copper was found to be bound exclusively to the respiratory pigment, haemocyanin; this labile association further allows copper to be past onto sites of accumulation following transport to the appropriate tissue (e.g. midgut gland) (Rtal and Truchot, 1996).

Nephrocytes are involved in endocytotic processes within the gills, removing cell debris (Smith and Ratcliffe, 1981), thus fulfilling an important function in keeping haemolymph channels unblocked and respiration physically unimpaired. They aid the "cleansing " of the crustacean haemolymph by detoxification as well as filtration and regulation of haemolymph components (Doughtie and Rao, 1981). Cell counts within anterior and posterior gill tissue from the different sample groups revealed no significant differences, although posterior gills generally contained significantly more nephrocytes than anterior gill tissue. One reason for this inter-gill bias in nephrocyte distribution may be related to the difference in lamellar size between the anterior and posterior gills relative to nephrocyte size. Accumulations of nephrocytes, which can reach up to 50 μm in diameter (Taylor and Taylor, 1992), would lead to blockages of the haemolymph channel, which in anterior gill lamellae may only be 15 μm wide. Such blockage would be detrimental to the physiological functioning of the gill by impairment of haemolymph flow. Similar observations of nephrocyte distribution within a posterior gill of *C. maenas* were made by Lawson (1994). Nephrocytes were rare in the distal gill lamellae (which are relatively small), but abundant in the larger mid and proximal gill lamellae (Lawson, 1994). It is, therefore, likely that the distribution of nephrocytes is dependent upon their requirement for space. The prevalence of nephrocytes in the proximal gill regions of the posterior gill, however, may be related to their role as the first line of defence against toxic substances. Their location coincides with the site of greatest ventilatory currents (Lawson,

1994) and the entry site of haemolymph to the gill via the infrabranchial sinuses (Taylor and Taylor, 1992). Finally, the biased distribution of nephrocytes may be due to functional differences between gill types and lamellae. Lawson (1994) proposed a predominantly respiratory function for distal lamellae within the posterior gill due to the dominance of chief cells in the epithelial layer. This suggestion corresponds to the functional specificity of anterior gills as a whole. However, since respiratory function and small lamellar size always occur together (and nephrocytes are not implicated as participants in gaseous exchanges), the size restriction is most likely to be the cause of nephrocyte distribution. The variability in size noted among nephrocytes in both gill types may be an artefact of different sectioning planes. The variability in internal appearance of the nephrocytes may be a reflection of their functional stage, i.e. more densely packed vacuoles being indicative of greater involvement of the cell in phagocytic activity. Johnson (1980; *Callinectes sapidus*) and Doughtie and Rao (1981; *Palaemonetes pugio*) linked the increase in vacuolar cell debris to an increase in time since the last moult. In the gills of intermoult *C. maenas*, however, nephrocytes with a wide range and density of contents were observed, indicating that nephrocyte content was not dependent on moult stage (see also Lawson *et al.*, 1994), although different amounts of cellular material within the cells may be a reflection of variation in the age of nephrocytes.

The epithelial cell type found predominantly in the posterior gills of *C. maenas* is the striated cell. These cells possess extensively folded apical and basal membrane systems, a common indicator of an ion transport function in cells throughout the animal kingdom (Berridge and Oschman, 1972). This feature leads to the distinction between osmoregulatory and respiratory gill epithelia in *C. maenas* (Copeland and Fitzjarrell, 1968; Compère *et al.*, 1989). The main distinguishing characteristic of these iono- and osmoregulatory striated cells is the presence of a highly infolded basal membrane system, each fold generally containing elongated mitochondria. This close association between elongate mitochondria and adjacent basolateral infoldings has been entitled "mitochondrial pumps" (Copeland and Fitzjarrell, 1968). In these epithelial cells, basal membranes are the location of ion exchange pumps, having high levels of Na,K-ATPase activity (Towle and Kays, 1986; Taylor and Taylor, 1992), the enzyme principally catalysing the exchange of ions (Siebers *et al.*, 1982). Stereological determination of the number of basal membrane infoldings, as well as associated mitochondrial area volumes ($V_{V/mit}$) (i.e. the relative area of the tissue section taken up by mitochondria), provided a theoretical estimate of the metabolic activity level of these cells. Results indicated a significant difference between

the number of basal infoldings in striated cells from Avon Estuary and Restronguet Creek crabs, the latter having a lower average number of infoldings (without any corresponding differences in apical membrane fold counts). As there was no sign of obvious membrane damage due to increased vacuolation [as shown by Nonnotte *et al.* (1993) and Lawson *et al.* (1995) following laboratory exposure to copper], this difference in membrane patterns appears to be population specific; the osmoregulatory membrane exchange surfaces in gills of crabs from the Avon Estuary being more elaborately folded than those from Restronguet Creek. Whether this is a reflection of an overall larger membrane surface area remains unclear without detailed measurement of the length of each membrane fold. Lawson (1994) reported a simultaneous lengthening of membrane folds with an increase of the total number of folds following exposure of *C. maenas* to dilute seawater. The morphological differences between these populations of crabs may, therefore, be salinity induced. Despite the higher membrane fold counts in striated cells in posterior gills of crabs from the Avon Estuary, there was no significant difference in the corresponding mitochondrial area volume ($V_{V/mit}$) of the cells, indicating that the ratio of mitochondrial area to the number of associated basal membrane infoldings differed between the groups. Striated cells from caged animals expressed the highest ratio of mitochondrial area volume attributed to each membrane fold compared with striated cells of crabs from the other two groups, and there appears to be a natural variation in the number of membrane folds associated with a particular mitochondrial area volume. A higher ratio may indicate a higher (theoretical) level of mitochondrial energy production for the same number of infoldings of the ion exchange membrane. It is likely, however, that there is a correlation between the actual membrane surface area (i.e. the area available for the location of ion exchange pumps) and mitochondrial area volume rather than between the number of membrane folds. This was not assessed quantitatively and, although it appears that the depth of fold is related to the height of the cell as a whole (the latter being approximately the same in all sample groups), it seems likely that all tissues from the three sample groups possess a similar ratio of basal membrane surface area to mitochondrial area volume. Unless actual tissue damage is apparent (in which case the ratio of mitochondrial area volume and basal membrane folds will prove a useful tool in the quantification of overt tissue damage), the ratios are a reflection of differences in the length of membrane infoldings rather than differences in activity levels. Gilles and Péqueux (1981) observed a change in the appearance of the apical membrane foldings with salinity adaptation, suggesting a direct relationship between salinity adaptation and cellular appearance, in

which apical membrane folds lengthen with increasing adaptation to low-salinity water. Similar claims regarding apical and/or basal membranes have also been made for other crustaceans (Lockwood *et al.*, 1973; Milne and Ellis, 1973; Foster and Howse, 1978).

The epithelial cell type characteristic of respiratory gill epithelia is the chief cell. Its large overall surface area, as well as the degree of apical membrane folding, makes chief cells well suited as the main site of gaseous exchanges, although the even thinner epithelial layers of the marginal canals have also been implicated as a major site for gaseous exchange (Taylor and Taylor, 1992). The ultrastructure of the respiratory epithelial surfaces was unaffected in caged crabs. This is inconsistent with laboratory exposures to trace metals, where ultrastructural changes were linked to physiological alterations (physiological measurements of gill function of caged crabs are necessary to confirm this assumption) (Nonnotte *et al.*, 1993; Lawson *et al.*, 1995; Chapters 4 to 6).

Pillar cells occur in posterior and anterior gill lamellae, connecting both sides of the lamella across the haemocoel. Their characteristic form in producing "pillars", together with the presence of extensive microtubular strands within the cytosol, implicate pillar cells as being (partly) responsible for direction of haemolymph flow and giving structural support to the gill lamellae (Taylor and Taylor, 1986). Significantly higher numbers of apical membrane infoldings were observed in pillar cells of the posterior gills of Avon Estuary compared with Restronguet Creek and transplanted crabs. Possible reasons for the relatively lower number of infoldings in caged and Restronguet Creek crabs differed. In the case of Restronguet Creek animals, the difference in the degree of apical folding in posterior gill pillar cells seemed to be due to inter-population differences as there was no sign of any obvious morphological damage. Changes in the degree of apical vacuolation, however, explain the depressed counts in tissues of caged crabs. An increase in apical vacuolation in striated cells has been reported previously as a consequence of exposure to reduced salinity (Lawson, 1994). Such an increase in vacuolation was due to the increase in the size (and frequency) of pinocytotic vacuoles (Lawson, 1994), but has also been observed as a consequence of exposure of *C. maenas* to water-borne trace metals (Lawson *et al.*, 1995). Although vacuolation did not appear to be extensive enough to affect the rooting of microtubular filaments in the cuticle, the strength of attachment of the pillar cells to the cuticle is likely to rely also on the many plasmalemma folds in contact with the endocuticle (Goodman and Cavey, 1990). It may be assumed that the structural strength of the pillar cells is not compromised in terms of the microtubular strands by the exposure of Avon Estuary animals to a new environment. Interruption of the apical membrane system,

however, my impair the function of this exchange surface, as both gaseous as well as ionic exchanges may not be exclusively carried out by chief and striated cell membranes (Taylor and Taylor, 1992), and lead to a detachment of pillar cells from the cuticle. Anterior gill pillar cells showed significant differences only in mitochondrial area volume, values being significantly higher in tissue from caged animals. This difference in measured area appears to be related to the occurrence of some swollen mitochondria which artificially increased the percentage determination of mitochondrial area within the tissue.

Two hypotheses are possible regarding the variation in cuticle thickness between the anterior and posterior gills. Firstly, cuticle thickness varies according to the size of the lamella, the smaller anterior gill lamellae being coated with a thinner cuticle than the larger posterior gill lamellae. This observation has been reported for lamellae of different location within the same gill (Lawson *et al.*, 1994). A positive correlation between cuticle thickness and relative size of adjacent epithelial cells may result in equal ratios between diffusion and metabolic processing of incoming gases and ions in lamellae of unequal size. Alternatively, size differences between lamellae reflect functional specificity. A relatively thinner cuticle may represent a prerequisite for successful respiratory gill function, whereas a thicker cuticle is present in osmo-/ionoregulatory gills. This hypothesis is supported by a decrease in the thickness of the cuticle toward and within the marginal canal (Lawson, 1994), the latter being implicated as an important site for gaseous exchanges within the gill (Taylor and Taylor, 1992). Gill cuticle thickness did not vary in animals between sites, and similar differences in cuticle thickness between gills of Avon Estuary and caged animals emphasised that this parameter was not affected by exposure to the environmental conditions of Restronguet Creek.

Measurement of the thickness of the total cuticular layer, including the algal/bacterial surface layer, revealed that there were differences in the amount and composition of this "flora", between sample groups and gill types from the same group. The presence of a gill flora has been reported often in decapods (Goodman and Cavey, 1990; Dalla Venezia *et al.*, 1992; Lawson *et al.*, 1994) and has been shown to be a source of iron to the gill (Johnson, 1980). The cuticle and any attached algal/bacterial flora are shed during the moult. To allow a valid comparison of the surface cover between gills, therefore, all sampled animals must be at intermoult stage. Both the anterior and posterior gill cuticles of the Avon Estuary crabs had relatively little adherent material. The posterior gill cuticle of Restronguet Creek crabs, however, often supported a double layer of algae (a very compact and uniform layer of algal-type cells overlaid with a thin layer of detrital

matter and another more diverse layer of algal cells). This difference in the cuticle "biofouling" may be related to differences in the sediment types between the two collection sites. The Avon Estuary contains sandy sediment whereas Restronguet Creek has a fine muddy sediment. Such differences in substratum may lead to differences in the algal microflora. Posterior gill cuticle of caged crabs had intermediate thicknesses of algal and detrital surface deposits (anterior gill cuticle, also, having a thicker layer in caged animals). The gill chambers of the transplanted crabs had a "dirty" appearance, all gills being partially coated in fine particles, whereas gill chambers of resident Restronguet Creek crabs appeared uncontaminated with such particles. The latter may be a reflection of a behavioural adaptation of the Restronguet Creek crabs, making greater use of the sweeping action of the epipodite of the first maxilliped to keep the gills free of sediment. Animals accustomed to the coarser sediment of the Avon Estuary may not to be able to respond to the finer sediment characteristics of their new environment. The deposits covering the gills at the time of dissection, however, may not be a true representation of their appearance in the live animal, as tissue fixation processes are likely to lead to the detachment of particles which are not securely fastened to the cuticle surface. Therefore, the algal/bacterial layer thickness calculated is merely a reflection of the amount of algae and detritus strongly adhering to the cuticle (either directly or indirectly). As yet, no detailed study of these surface layers on crustacean gills has been carried out. There is, therefore, no information available on the species composition or any dependence thereof on seasonal availability of particular algae or bacteria in the surrounding water.

In conclusion, there were differences in the cellular morphology between the gills of crabs living in the Avon Estuary and Restronguet Creek. These differences do not appear to represent gill damage but imply different functional adaptations of the gill tissue to their environment. It is not yet determined whether these differences are related to the degree of exposure to trace metals in the two environments, although differences in tissue MT and total metal levels (as well as physiological performance) may support such a hypothesis. To elucidate the differences in gills from the two field sites, and any effects caused by temporary exposure of *C. maenas* to the contaminated site, analytical electron microscopical techniques must be applied, as for example reported by Lawson (1994) who used x-ray microanalytical techniques on both control and laboratory-exposed crabs to investigate the fate of copper within the gill epithelium. The appearance of some ultrastructural gill changes, together with an increase in MT concentrations, in transplanted

animals from a similar environment indicates that crabs respond to acute exposure to trace metals in the field. These findings further emphasise the limited relevance of laboratory exposures to a single contaminant, as laboratory exposure to copper at higher levels than present at the sample site in Restronguet Creek failed to cause any gill ultrastructural damage (Chapter 3). Although this study emphasised metal contamination of the Fal Estuary, Restronguet Creek was classified as an estuary of "poor biological water quality" in the 1995 Environment Agency General Quality Assessment. Animals living in this environment are, therefore, also exposed to raised ammonia levels from the contributory River Carnon. The combination of different metals and other contaminants has to be considered in the evaluation of toxic effects. At least phenotypic gill responses of crabs permanently "resident" in Restronguet Creek appear to have occurred, which is supported by reports of cellular recovery during prolonged exposure to copper in the laboratory, following initial acute gill damage (Nonnotte *et al.*, 1993).

Figure 7.1: a-b. Position of the Avon Estuary (A) and the Fal Estuary (B) within the UK. **c.** Position of the "control" sampling site (arrow) in the Avon Estuary (Devon). **d.** Position of the "polluted" sample site and location of transplantation cage (arrow) in Restronguet Creek, Fal Estuary (Cornwall).

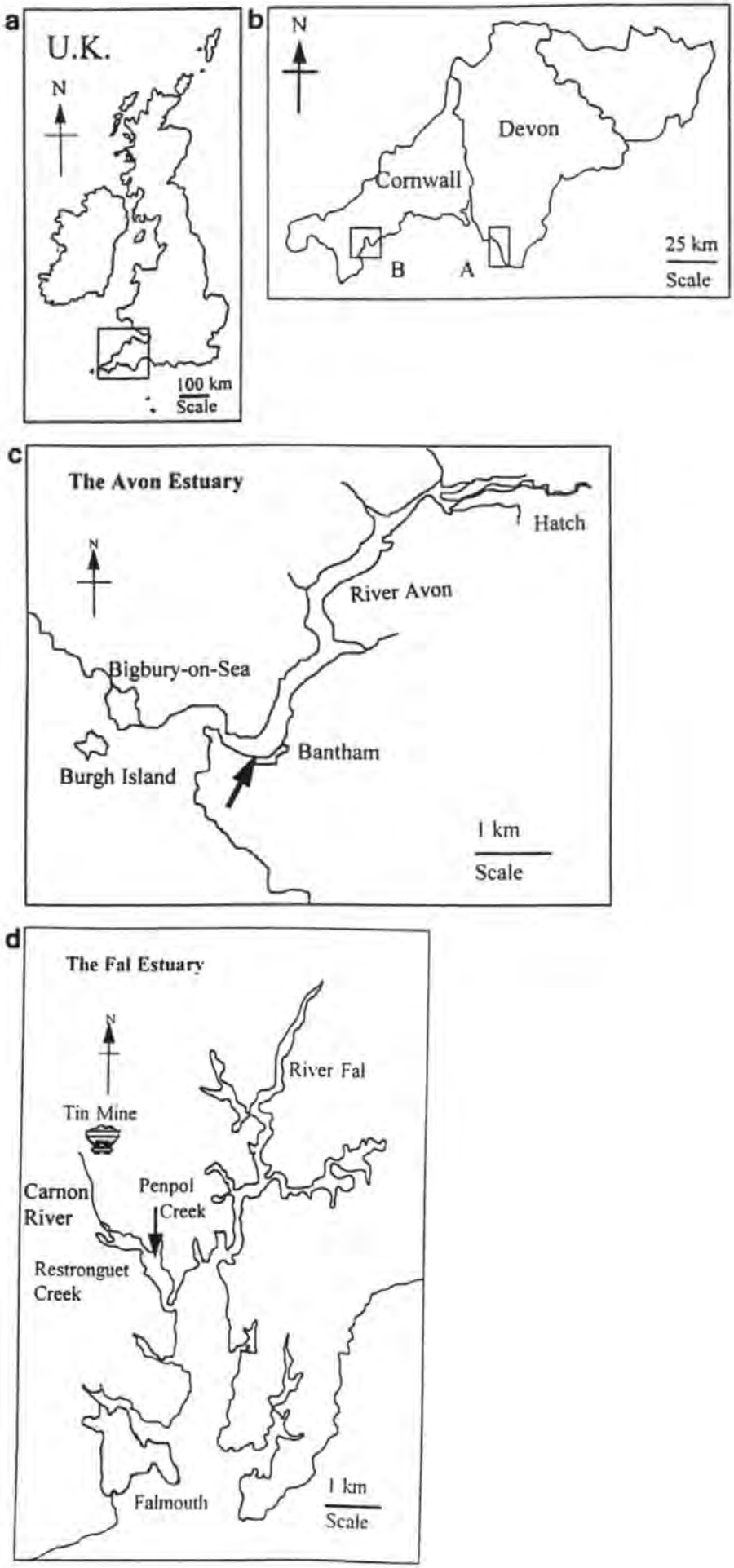

a
U.K.
N
100 km
Scale
b
N
Devon
Cornwall
B
A
25 km
Scale
c
The Avon Estuary
N
Hatch
River Avon
Bigbury-on-Sea
Bantham
Burgh Island
1 km
Scale
d
The Fal Estuary
N
River Fal
Tin Mine
Carnon
River
Penpol
Creek
Restronguet
Creek
Falmouth
1 km
Scale

Figure 7.2: Sample site in Avon Estuary (near Bantham, Devon). **a.** West toward river mouth. **b.** North East up river.


a


b

Figure 7.3: a. Position of sampling site and position of transplantation cage in Restronguet Creek (looking North West up Carnon River). **b.** Transplantation cage, partially buried in sediment, with stainless steal mesh walls and perspex lids to 8 individual chambers.


a


b

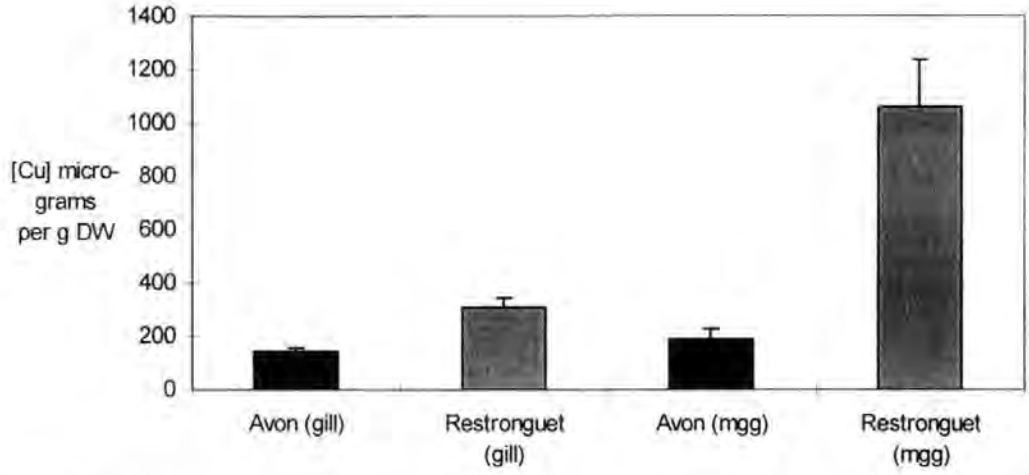


Figure 7.4: Gill tissue Cu levels [μg Cu g^{-1} dry weight (DW)] measured in crabs from the Avon Estuary (control site) and Restronguet Creek (polluted site) (means with standard error bars). [n=5 in each sample]

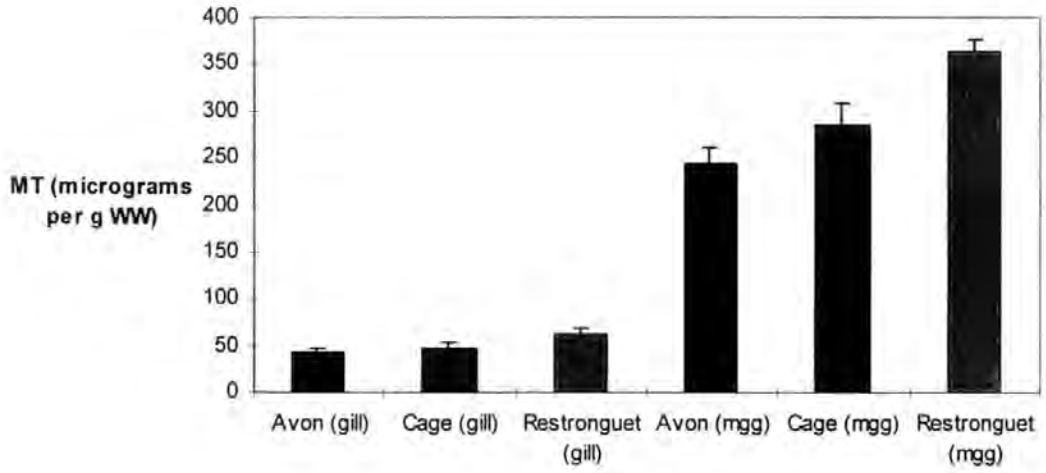
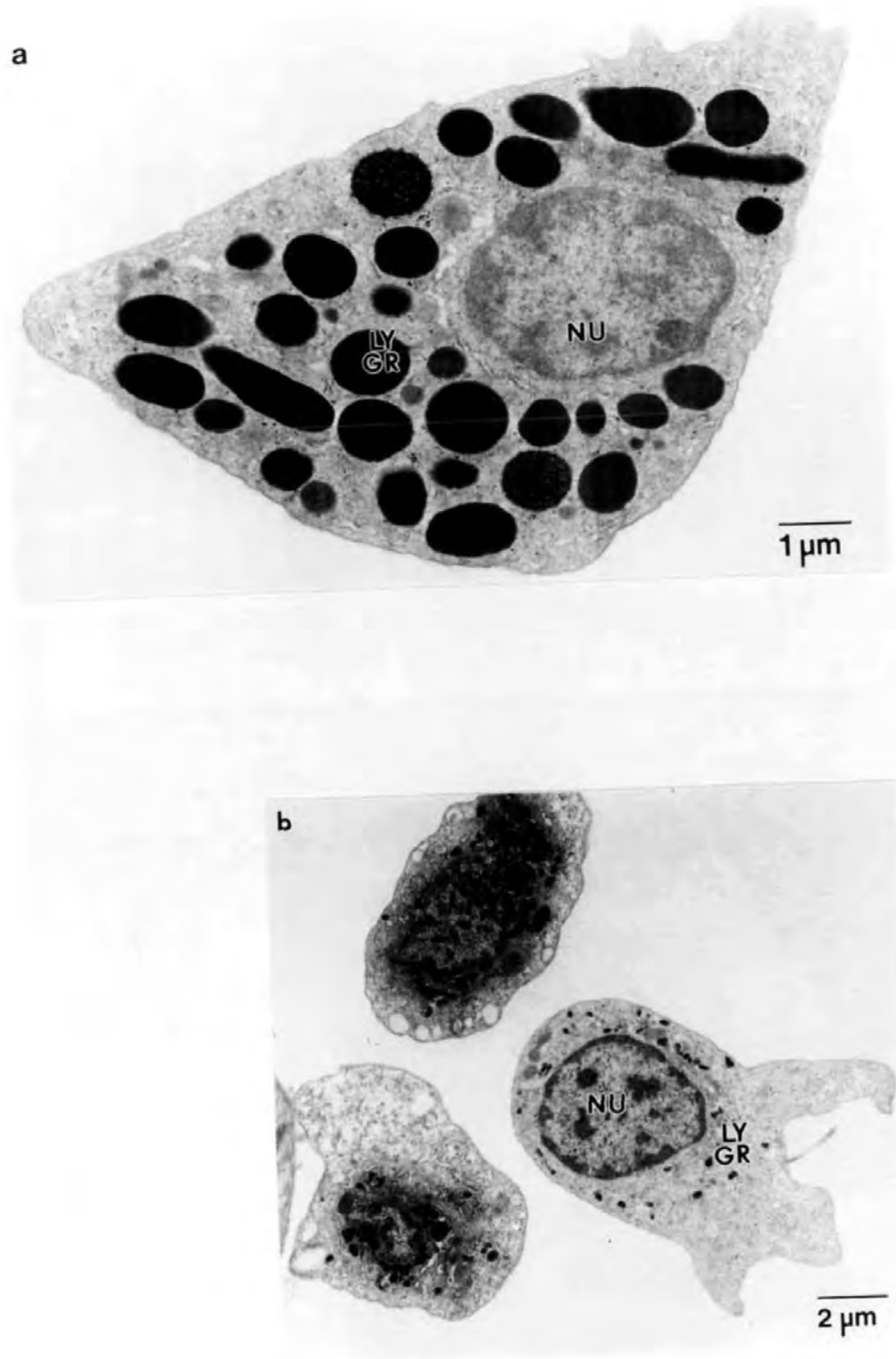


Figure 7.5: Gill and midgut gland (mgg) tissue metallothionein (MT) concentrations [μg g^{-1} wet weight (WW)] measured in crabs from the control (Avon Estuary) and the polluted Restronguet Creek) sites, and the transplant experiment (cage) (means with standard error bars). [Avon Estuary (gill), n=15; Avon Estuary (mgg), n=3*; Cage (gill/mgg), n=7; Restronguet Creek (gill/mgg), n=8]

*remaining samples were lost

Figure 7.6: Haemocytes in the gill tissue of *Carcinus maenas*. **a.** Large-granule haemocyte. **b.** Small-granule haemocyte. *GR*, granule; *LY.*, lysosome; *NU*, nucleus.


a
LY
GR
NU
1 µm
b
NU
LY
GR
2 µm

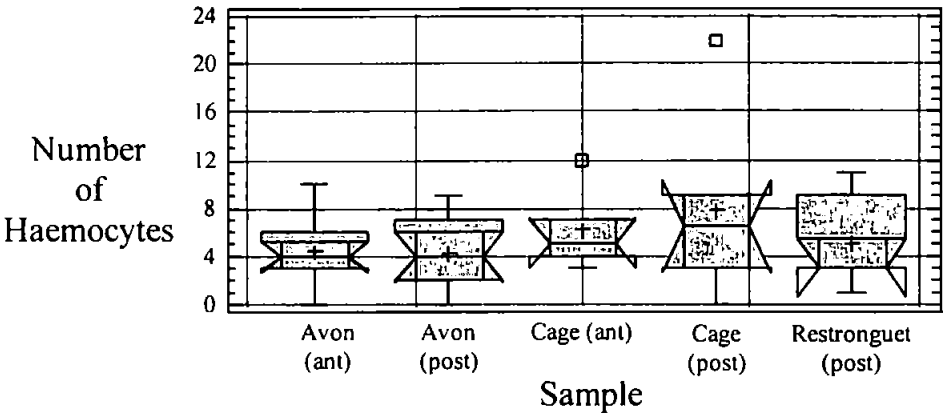
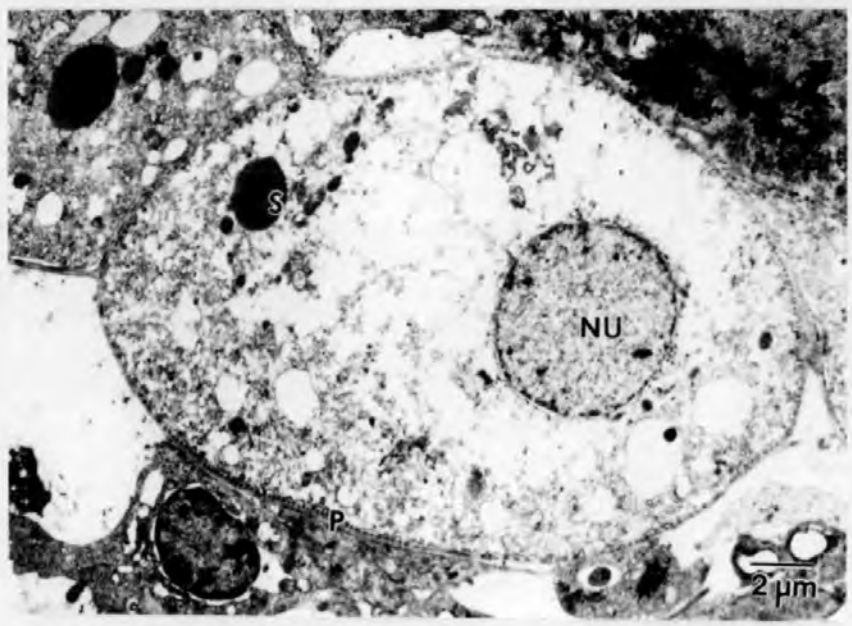


Figure 7.7: Box-Whisker plot* showing the number of haemocytes (per 300μm of section) counted at TEM level in anterior (ant) and posterior (post) gill tissue of Avon Estuary, transplanted (Cage) and Restronguet Creek (posterior gill only) animals. [Avon (ant) and Avon (post), n=13; Cage (ant), n=4; Cage (post), n=6; Restronguet (post), n=11]

* see Appendix 3

Figure 7.8: Nephrocyte in gill tissue of *Carcinus maenas*. *NU*, nucleus; *P*, pedicel; *S*, satellite vacuoles.


S
NU
P
2 µm

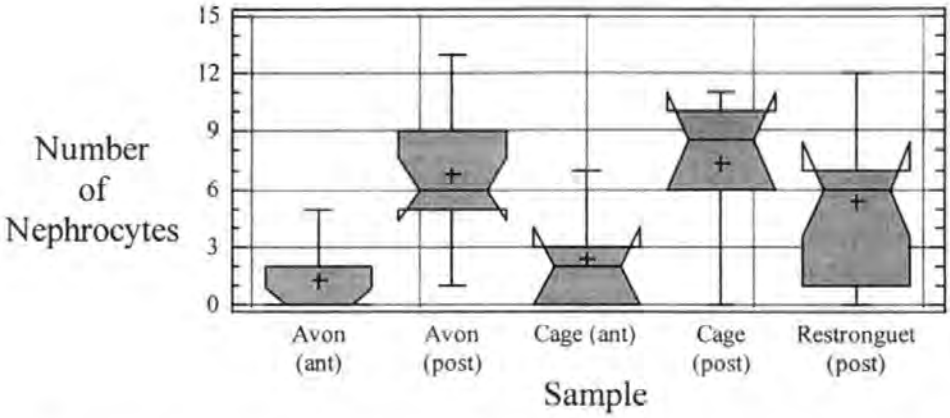
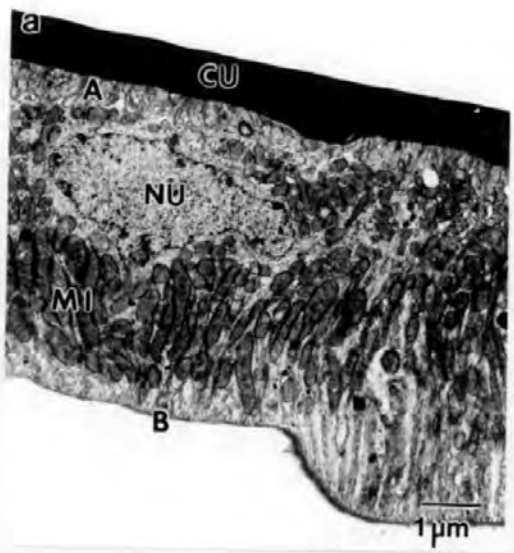
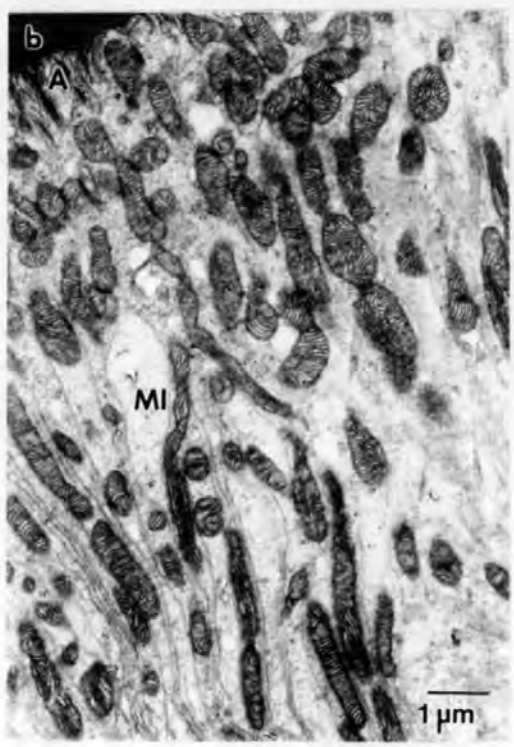


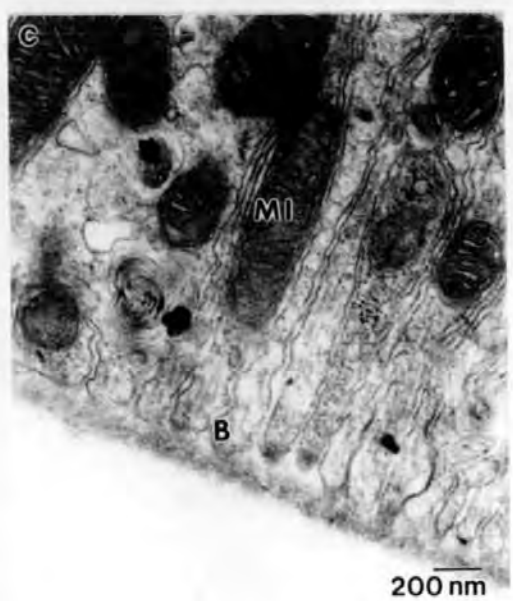
Figure 7.9: Box-Whisker plot* of the number of nephrocytes (per 300μm of section) counted at TEM level in anterior (ant) and posterior (post) gill tissue of Avon Estuary, transplanted (cage) and Restronguet Creek (posterior gill only) animals [Avon (ant) n=13; Avon (post) n=14, Cage (ant), n=4; Cage (post), n=6; Restronguet (post), n=11].

* see Appendix 3

Figure 7.10: a. Striated cell in the gill epithelium of *Carcinus maenas*. **b.** Apical membrane of striated cell, with elongate mitochondria between membrane folds of the basal lamina. **c.** Basal membrane infoldings in striated cells with elongate mitochondria, forming "mitochondrial pumps". *A*, apical membrane; *B*, basal membrane; *CU*, cuticle; *MI*, mitochondria; *NU*, nucleus.


a
CU
A
NU
MI
B
1 µm


b
A
MI
1 µm


c
MI
B
200 nm

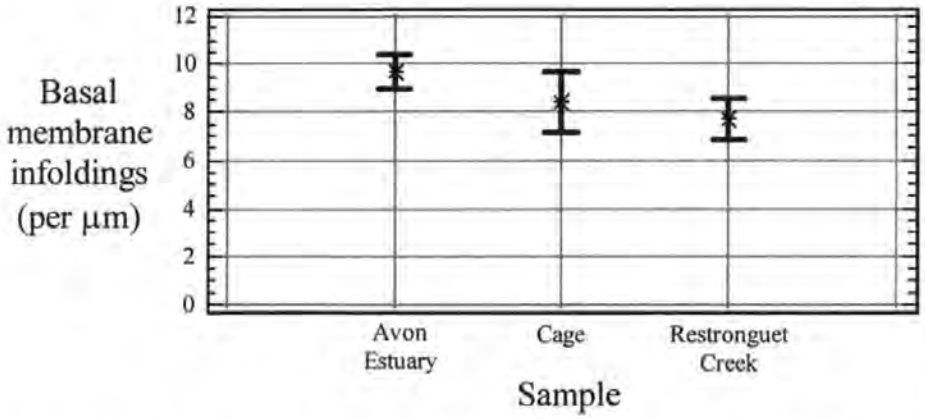


Figure 7.11: Number of basal membrane invaginations (per μm) in striated cells of posterior gill samples (means with 95% least significant difference (LSD) intervals). [Avon, n=12; Cage, n=6; Restronguet, n=14]

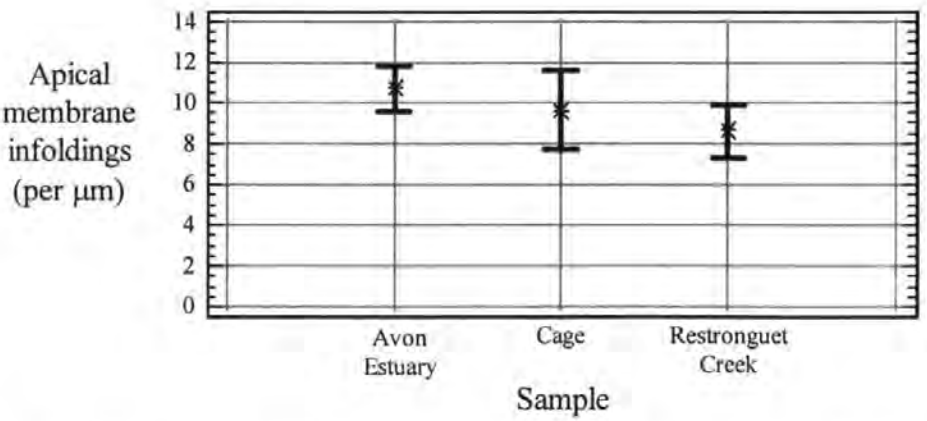


Figure 7.12: Number of apical membrane invaginations (per μm) in striated cells of posterior gill samples (means with 95% least significant difference (LSD) intervals). [Avon, n=12; Cage, n=6; Restronguet, n=14]

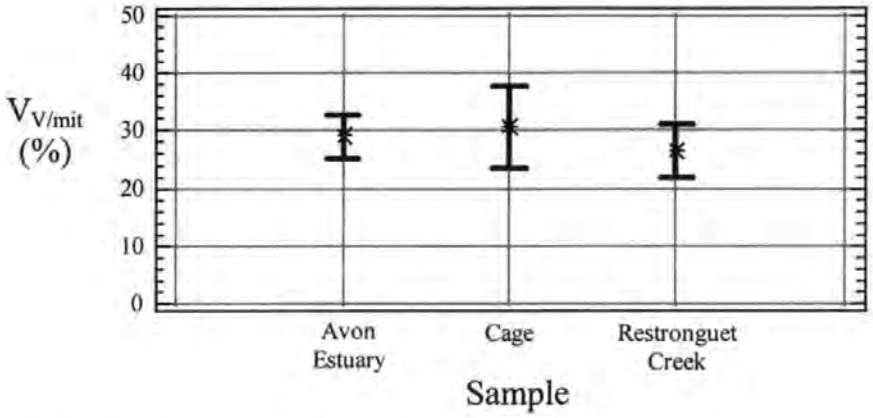
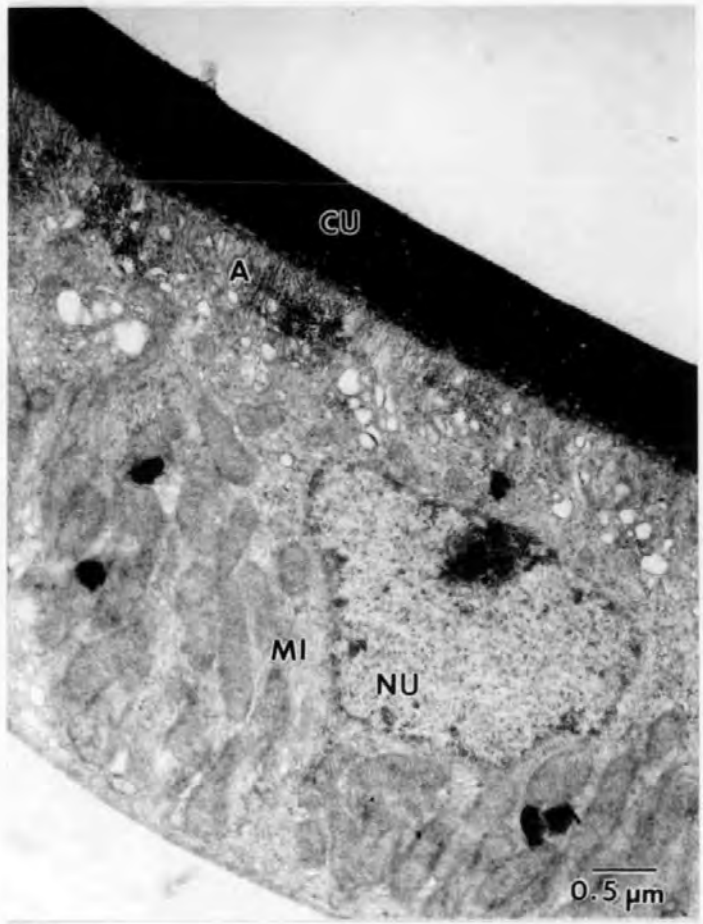


Figure 7.13: Mitochondrial area volumes ($V_{V/mit}$) in striated cells of posterior gill samples (means with 95% least significant difference (LSD) intervals). [Avon, n=12; Cage, n=6; Restronguet, n=14]

Figure 7.14: Chief cell in gill epithelium of *Carcinus maenas*. *A*, apical membrane; *CU*, cuticle; *MI*, mitochondria; *NU*, nucleus.


CU
A
MI
NU
0.5 µm

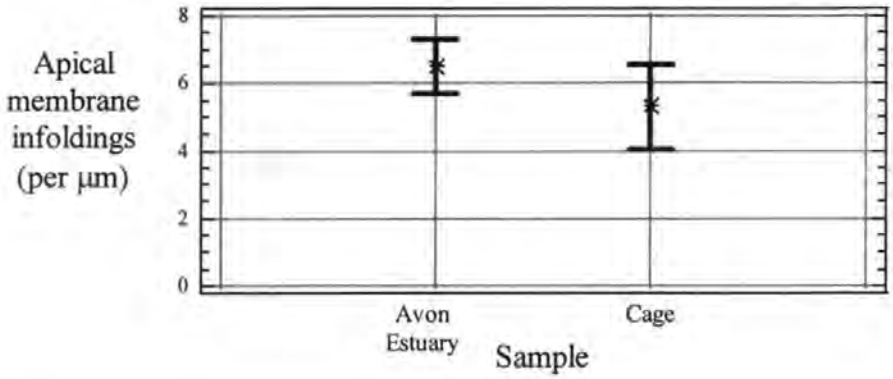


Figure 7.15: Apical membrane invaginations (per μm) in chief cells of anterior gill tissue from Avon Estuary and transplanted (cage) animals (means with 95% least significant difference (LSD) intervals). [Avon, n=15; Cage, n=6]

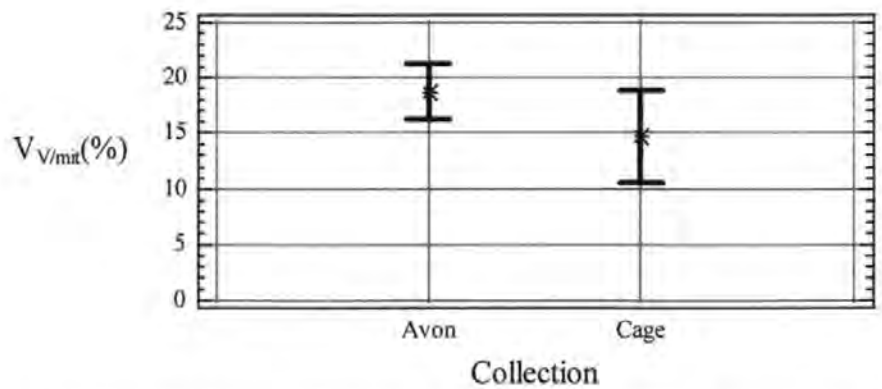
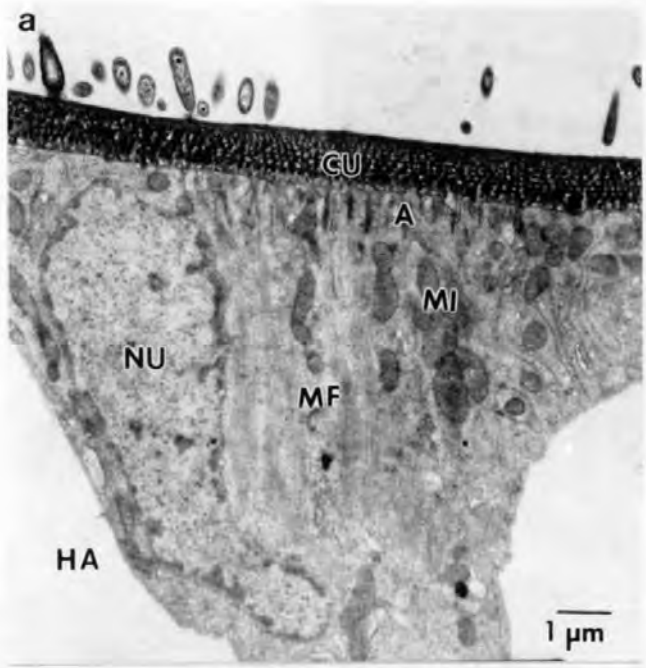
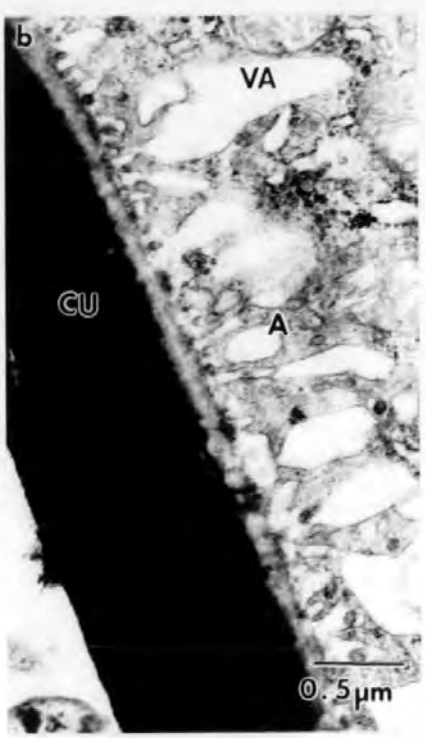
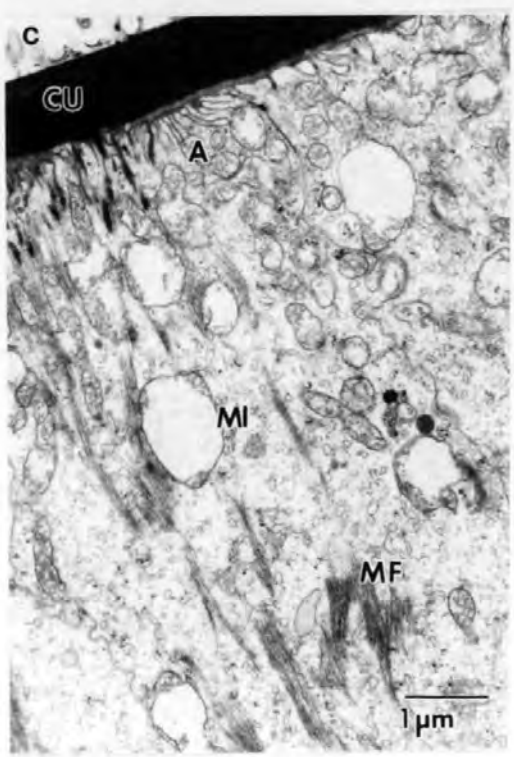


Figure 7.16: Mitochondrial area volumes ($V_{V/mit}$) in chief cells of anterior gill lamellae of Avon Estuary and transplanted (cage) animals (means with 95% least significant difference (LSD) intervals). [Avon, n=15; Cage, n=6]

Figure 7.17: a. Pillar cell in gill epithelium of *Carcinus maenas*. **b.** Apical membrane of pillar cell in posterior gill epithelium of a transplanted crab, showing increased vacuolation. **c.** Cytoplasm of pillar cell of an anterior gill in transplanted animals, showing swollen mitochondria. *A*, apical membrane; *B*, basal membrane; *CU*, cuticle; *HA*, haemocoel; *MF*, microfilaments; *MI*, mitochondria; *NU*, nucleus; *VA*, vacuole.


a
CU
A
MI
NU
MF
HA
1 μm


b
VA
CU
A
0.5 μm


c
CU
A
MI
MF
1 μm

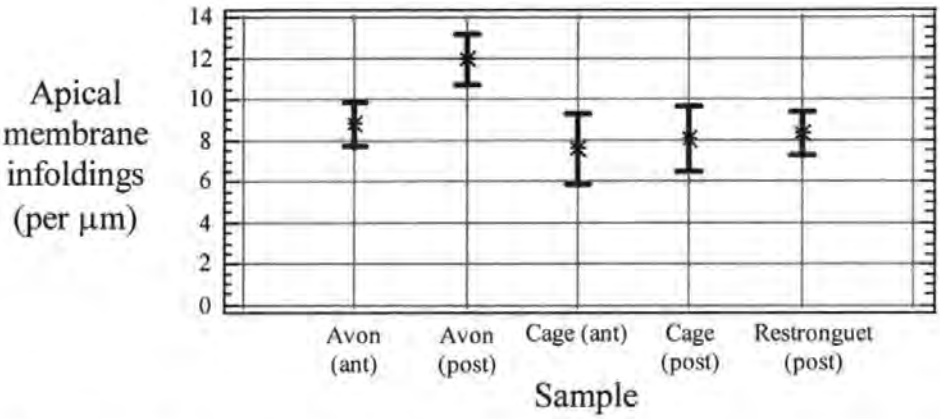


Figure 7.18: Apical membrane infoldings in pillar cells of anterior (ant) and posterior (post) gill tissue of Avon Estuary, transplanted (cage) and Restronguet Creek (posterior gill only) animals (means and 95% least significant difference (LSD) intervals). [Avon (ant) n=13; Avon (post) n=10, Cage (ant), n=5; Cage (post), n=6; Restronguet (post), n=14].

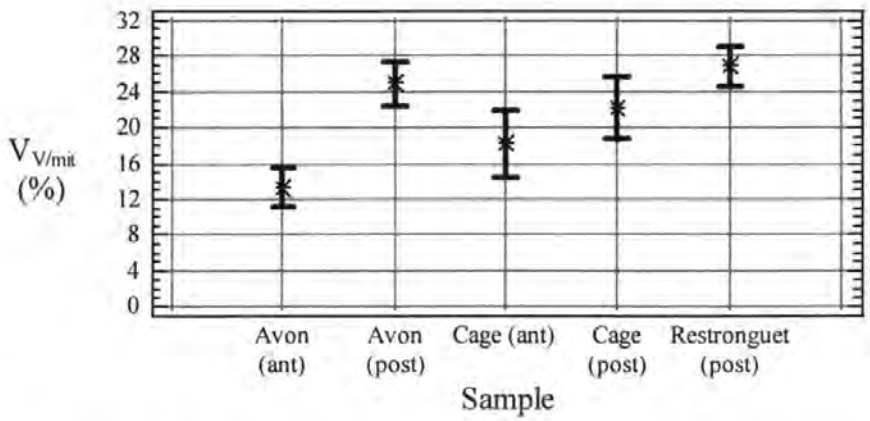


Figure 7.19: Mitochondrial area volumes ($V_{V/mit}$) in pillar cells of anterior (ant) and posterior (post) gill tissue of Avon Estuary, transplanted (cage) and Restronguet Creek (posterior gill only) animals (means and 95% least significant difference (LSD) intervals). [Avon (ant) n=15; Avon (post) n=12, Cage (ant), n=5; Cage (post), n=6; Restronguet (post), n=14].

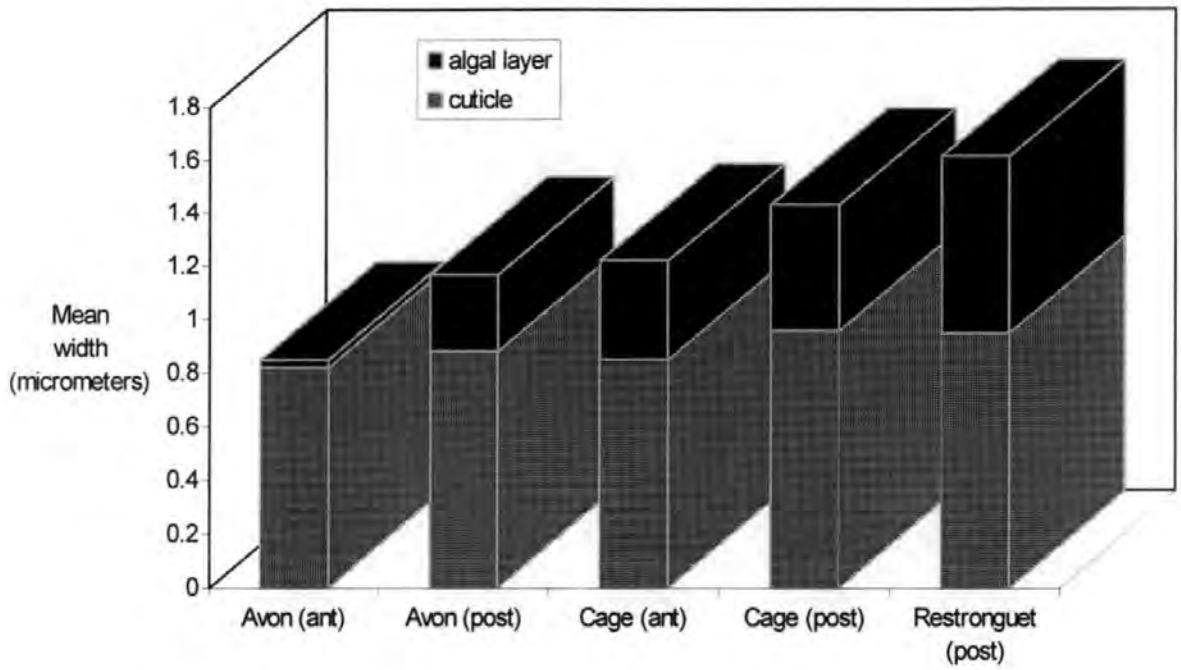
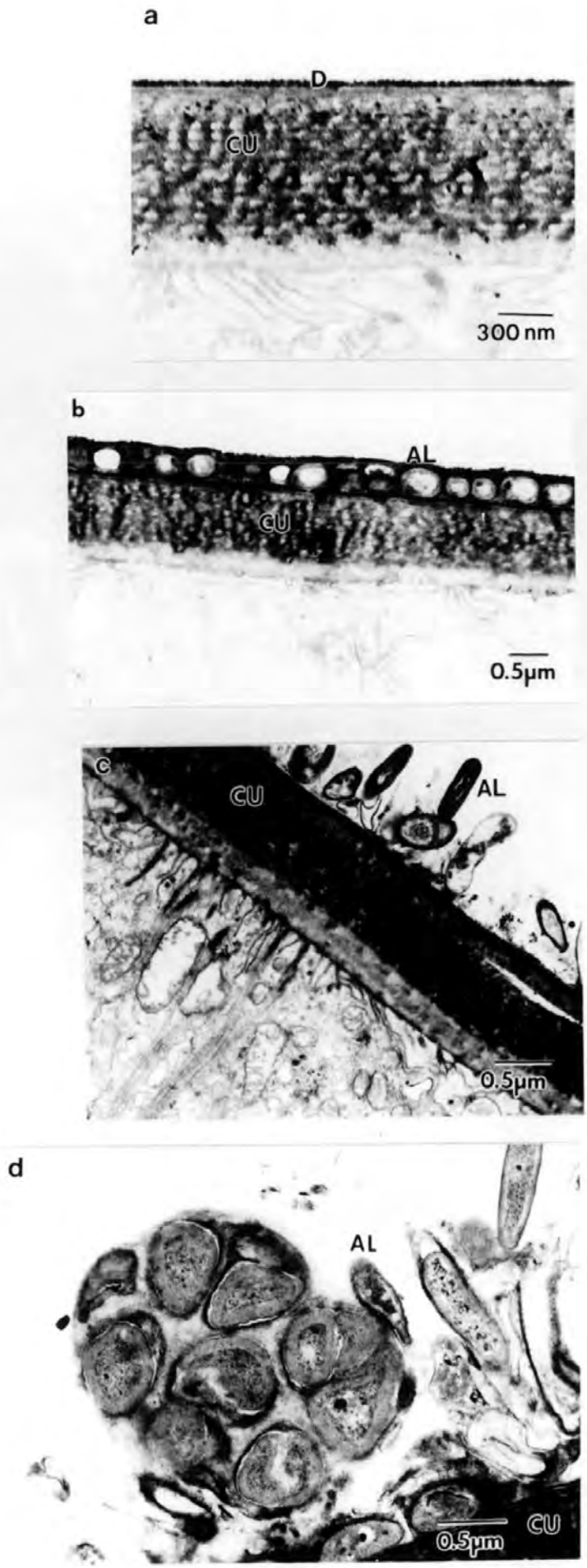


Figure 7.20: Mean width of total cuticular layer, illustrating proportion of actual cuticle and algal/bacterial surface layers in anterior (ant) and posterior (post) gills, in animals from the Avon Estuary, Restronguet Creek and the transplant experiment. [n=11 (Avon/ant), n=5 (Cage/ant), n=14 (Avon/post), n=6 (Cage/post), n=14 (Restronguet]

Figure 7.21: a. Detrital layer on the anterior gill cuticle of a crab, *Carcinus maenas*, from the Avon Estuary. **b.** Monolayer of algae/bacteria on the cuticle surface of a posterior gill of a crab from the Avon Estuary. **c.** Diverse flora on an anterior gill of a transplanted animal. **d.** Multilayered algal/bacterial layer on the cuticle surface of a posterior gill of a crab collected from Restronguet Creek. *AL*, algal/bacterial layer; *CU*, cuticle; *D*, detrital layer.


a
D
CU
300 nm
b
AL
CU
0.5µm
c
CU
AL
0.5µm
d
AL
0.5µm
CU

CHAPTER 8

GENERAL DISCUSSION
AND
CONCLUSIONS

This study has contributed to the understanding of the effects of copper exposure on the shore crab *Carcinus maenas* by providing an integrated view of contaminant effects at three levels of biological organisation, incorporating cellular, physiological and molecular aspects.

At the cellular level, it was established that gill ultrastructure does not reflect low-level (50 μg L^{-1}) copper exposure in the laboratory. This conclusion, reached following exposure in a flow-through and a static seawater system, was unexpected as an earlier study, using the same flow-through system, had reported changes in the ultrastructure of a posterior gill of *C. maenas* at this copper concentration (Lawson *et al.*, 1995). The experimental animals used in the two studies were taken from different field sites, and this may explain the different responses. Although the sample sites are relatively close, Lawson *et al.* (1995) took animals from a marine site, whereas the crabs collected for the present study were taken from an estuarine site. The relative sensitivity of marine and estuarine animals to contaminant exposure is an ongoing debate. Some workers propose that estuarine species living at the extremes of their tolerance range are sensitive to any added stress (Jones, 1973). In contrast, Gray (1974) believes that estuarine species, adapted to the environmental changes encountered, have an increased tolerance to contaminant exposure. In the current study, the lack of ultrastructural response to low-level copper exposure in the laboratory may be explained partly by a relative insensitivity of the gills of *C. maenas* to copper at that concentration (possibly related to their estuarine habitat), and partly by the natural variability in the appearance of gill epithelia which may mask any subtle changes in gill ultrastructure following copper exposure. The naturally occurring variability in cell condition underlines the need for a quantitative approach in comparative ultrastructural studies (unless cellular changes are very pronounced). For future studies, therefore, the use of morphometric techniques (e.g. including stereology and image analysis methods) is recommended. The availability of quantitative data would allow comparisons with other studies, minimising the risk of subjective observations, e.g. due to the analysis of only few samples as generally found in qualitative studies. Indeed, this danger is highlighted by the comparison of observations of nephrocyte numbers following two exposures to 500 μg Cu L^{-1} in this study. An increase in nephrocyte numbers was reported following a qualitative assessment of samples (Chapter 2). A quantitative determination of nephrocyte numbers following exposure to the same level of copper (Chapter 5), however, contradicted this observation. This contradiction may be caused by differences in exposure duration or

physiological condition of the animals, however, it seems more likely, that this difference represents an example of the dangers of relying on the qualitative analysis of a small number of samples. With the development of new image analysis techniques which can be connected directly to the (transmission) electron microscope, cost and analysis time required, which formerly may have curtailed the feasibility of quantitative studies, are minimised.

By exposure of *C. maenas* to various concentrations of copper in a static seawater system, the threshold level for the initiation of cellular change was established. The ultrastructure of the smaller, anterior (respiratory) gills was far more sensitive to copper exposure than the larger, posterior (osmoregulatory) gills. The former showed first signs of "damage" at 100 μg Cu L^{-1}, whereas the latter remained unaffected until exposure to 300 μg Cu L^{-1}. Even at the highest copper concentration (500 μg L^{-1}), the posterior gill was not so severely impacted as the anterior gill. The general conclusion, therefore, is that respiratory epithelia are more sensitive to copper exposure than osmoregulatory epithelia. The apparently higher tolerance of the osmoregulatory epithelia (*cf.* respiratory epithelia) to copper in *C. maenas* may be due to their role in the exchange of ions and essential trace metals through pinocytosis, diffusion and exchange pumps (Rainbow and Dallinger, 1993). The participation of enzyme-mediated transfer in metal uptake in the posterior gills of *C. maenas* has been demonstrated recently, and is estimated to account for approximately 30% of total uptake, the remaining 70% entering by passive means (Clausen *et al.*, 1993). Osmoregulatory tissues will be able to process exogenous copper and remain undamaged to a critical threshold. Above this threshold, enzymes involved in ionic exchanges (such as Na,K-ATPase and carbonic anhydrase) are inhibited, as shown by Hansen *et al.* (1992) with *C. maenas* and relatively high copper concentrations (10 mg L^{-1}). At 10 mg L^{-1}, Na,K-ATPase activity [the main enzyme responsible for osmoregulatory exchanges (Siebers *et al.*, 1982)] was inhibited and the ability to respond to environmental salinity changes impaired (Hansen *et al.*, 1992). Such a reduction in enzyme activity may be due to direct toxicity of the metal or to indirect effects such as a reduction of the membrane surface area containing the enzyme due to ultrastructural damage. Copper alters membrane structure by lipid peroxidation (Viarengo, 1985), which in return (probably due to the formation of oxyradicals) can cause degradation of enzymes, glycoproteins as well as DNA (Viarengo *et al.*, 1988). Lawson (1994) suggested that disruption of cell membranes may lead to reduced Ca^{+} transport from the cells. Such a cytosolic increase in Ca^{+} has been shown to be toxic (Nicotera *et al.*, 1989).

In contrast to the effects of copper on osmoregulatory epithelia, the respiratory epithelia of *C. maenas* are designed for the exchange of gases rather than solutes or metals. This function is mirrored by the very low levels of Na,K-ATPase in the tissue (Siebers *et al.*, 1982). Respiratory epithelia will accumulate copper more rapidly than osmoregulatory epithelia (as shown for cadmium by Clausen *et al.*, 1993), linked to their inability to remove the metal, e.g. by pinocytosis or active transport to the haemolymph. Tolerance of these epithelia to exogenous copper will be limited, as threshold levels of toxic effect are reached more rapidly. It would be interesting to investigate if this structurally expressed difference in sensitivity is expressed physiologically. This could, for example, be achieved by measuring haemolymph parameters associated with both ionic and respiratory exchanges of the gill epithelia.

Although there are numerous studies reporting the effects of trace metals on crustacean gill ultrastructure following laboratory exposures (Section 2.1), this study compared the gill ultrastructure of crabs from field sites with different levels of (waterborne) trace metal (Chapter 7). Morphometric analyses showed that there were differences in the structure of epithelial cells in animals from the two populations. These differences, however, did not correspond to the ultrastructural gill damage observed following acute laboratory trace metal exposure. The differences observed (varying densities of basal and apical membrane folds) may represent functional adaptations to the two environments. Animals transferred from the "clean" to the contaminated field site showed epithelial characteristics similar to those measured in the laboratory for *C. maenas* exposed to relatively low levels of copper. It was not possible to conclude if these structural responses were caused by metal levels or other environmental factors (e.g. a change in environmental salinity). A reciprocal transplant of animals should, therefore, be carried out to determine if animals from the contaminated field site showed the same ultrastructural changes when transplanted to the "clean" site. Such a transplant experiment would also elucidate if differences between the animals from the two field sites were due to phenotypic or genotypic adaptation (Vernberg, 1967). In conclusion, the absence of overt gill damage at the level of bioavailable copper present at the contaminated field site is in accord with the lack of ultrastructural change observed following laboratory exposure to slightly higher levels. This is surprising, as the mixture of trace metals encountered in the field may be expected to exert an additive (or synergistic) effect, although changes in

chemical complexation could account for this apparent lack of a toxic effect (Zamuda and Sunda, 1982; Zamuda *et al.*, 1985; Blust *et al.*, 1986; Bryan and Langston, 1992).

This study validated the use of the Computer Aided Physiological Monitor (CAPMON) (Andersen and Depledge, 1990) for the recording of scaphognathite activity in crabs (Chapter 4). Although the system has some disadvantages, compared with other methods of detecting ventilatory and cardiac activity in crustaceans (including the impedance technique and the pressure transducer), the ability of CAPMON to conduct physiological measurements completely non-invasively is a significant advance. One disadvantage is the fact that CAPMON records data as beats per minute. This limits the sensitivity of the recordings. For example, short periods of apnoea are missed. This problem can be countered by software adjustments to allow counts of scaphognathite beats within shorter time periods. An important failing of the CAPMON system is its inability to detect scaphognathite reversals. CAPMON detects scaphognathite movement from variations in the strength of the reflected infra-red beam emitted by the sensor, irrespective of the direction of the passage of the scaphognathite. It is difficult to envisage how this problem can be solved.

Exposure to copper, followed by changes in temperature and oxygen levels, lead to changes in the scaphognathite activity of *C. maenas*. In Chapters 4 and 5, the most striking change in the ventilatory activity of copper-exposed crabs was the significant reduction in scaphognathite rate. Although this contradicts observations following trace metal exposure in *Crangon crangon* (Price and Uglow, 1980), the reduction in scaphognathite rate is supported by reports of decreased oxygen consumption following trace metal exposure in other Crustacea (Spicer and Weber, 1991). In Chapter 6, copper-exposed *C. maenas* displayed a significant increase in scaphognathite rate, thus contradicting measurements in the two previous Chapters. The three sets of animals were collected at different times of the year (Chapter 4: January; Chapter 5: May; Chapter 6: November), suggesting that the response of *C. maenas* to copper exposure may, therefore, be related to variations in the physiological condition of the population. This variability in respiratory responses to trace metal exposure in crustaceans is illustrated in the literature (see review by Spicer and Weber, 1991). This study, therefore, casts further doubt on the usefulness of scaphognathite rate measurements as an assessment of contaminant effects in crustaceans.

The current study examined whether the added challenge of temperature or hypoxia for short time periods following prolonged exposure to copper would cause

different effects in *C. maenas*. Although these experimental conditions present a likely situation in the field, it would be interesting to assess the effects of prolonged exposure to copper at raised temperature/decreased oxygen. Damage to gill ultrastructure may be exacerbated by exposure to copper at a higher temperature, as increased metabolic rates may encourage trace metal uptake. Similarly, the level of hypoxia and temperature may affect the synthesis of metallothionein. Effects of temperature and hypoxia, linked to trace metal exposure, on gill ultrastructure and metallothionein detoxification offer a profitable line of future research.

The gill tissue metallothionein concentrations of *C. maenas* varied with no consistent pattern. Although the test organisms in Chapters 4 - 6 were exposed to the same concentration of copper under similar exposure conditions, the metallothionein concentrations were only elevated in gill tissue from crabs in Chapter 5 (collected in May). Similar variability in metallothionein measurements has been observed by other workers using the same quantification technique (Wedderburn, pers. com.; Astley, pers. com.). The recent study of Pedersen and Lundebye (1996) did not report significant differences in metallothionein concentrations in the midgut glands of animals from the same field sites as used here, yet this study did find significant differences (Chapter 7). Differences in metallothionein concentrations in gill tissue of *C. maenas* from the two field sites, however, did correspond between the present study and that by Pedersen *et al.* (1997). These differences in measurements may be due to the season in which animals were collected. Differences in the saturation of metal-binding sites in *C. maenas*, e.g. related to haemocyanin synthesis (Brouwer *et al.*, 1986; Engel and Brouwer, 1987), may be determining how rapidly metallothionein synthesis is initiated following the influx of exogenous copper. In the current study, metallothionein concentrations were measured in gill tissue which was severely damaged. As the synthesis of metallothionein depends on the functioning of certain cytoplasmic organelles (e.g. the endoplasmatic reticula), the variability in response may be linked also to the inability of the damaged tissue to synthesise these proteins. In summary, the differences in metallothionein response under the same exposure regimes may be linked to the speed at which metallothionein synthesis is initiated, which in turn is associated with the saturation of existing metallothionein in accordance with the physiological state of the crabs. Animals, therefore, may or may not be able to initiate the *de novo* synthesis of metallothionein prior to its inhibition due to cellular disintegration.

In Chapters 4 - 6, the response of *C. maenas* to copper exposure was assessed at three levels of biological organisation, including gill ultrastructure, scaphognathite activity and gill tissue metallothionein concentrations. Severe damage to gill ultrastructure occurred simultaneously with changes in scaphognathite activity and, in one of the three experimental groups, in combination with a significant increase in the concentrations of gill tissue metallothionein concentrations. These results show, that exposure to copper (for 10 days at 500 μg Cu L^{-1}), has a simultaneous impact at the cellular, physiological and molecular level of *C. maenas*. Considering the variability in response of metallothionein concentrations and scaphognathite activity, gill ultrastructure represents the most consistent indicator of copper exposure at this contaminant concentration. It may be argued that changes to cellular structures are not indicative of physiological impairment to the animals by contaminant exposure, particularly as the severity of ultrastructural gill "damage" (in Chapters 4 and 5) did not seem proportional to the compensatory change in saphognathite activity. In contrast, the significant differences between the scaphognathite activities of control and copper-exposed animals in Chapter 6 appear to mirror adequately the functional impairment of gills [measured in terms of haemolymph chemistry by Nonnotte *et al.* (1993)] suggested by the damaged gill ultrastructure.

In conclusion, gill ultrastructure (rather than scaphognathite activity or metallothionein concentrations) was found to consistently reflect exposure of *C. maenas* to copper in the laboratory. The suitability of qualitative ultrastructural studies to detect copper exposure is restricted to (in the case of gill tissue) exposure concentrations exceeding 50 μg Cu L^{-1}. Differing sensitivities of gill and epithelial types emphasises the need for careful selection of tissue samples. Ideally, all ultrastructural studies would use morphometric techniques to ensure objectivity and to allow valid comparisons between treatments, field samples and different studies. In general, although experiments involving laboratory exposures offer insights into the mechanisms of toxic action of trace metals, chronic exposures in the field, in combination with water chemistry variables influencing trace metal toxicity, are likely to affect experimental organisms differently to laboratory exposures.

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APPENDIX 1

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Responses of Crustaceans to Contaminant Exposure: a Holistic Approach

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Typically, the sublethal effects of exposure to environmental contaminants are assessed by measuring the responses of one, or few, biochemical or physiological systems within organisms. An individual's survival potential, however, is determined by the integrated phenotypic response of all its systems working in concert. This concept is illustrated here by reviewing the repertoire of responses at several levels of organization (from the molecular to the behavioural) available to the common shore crab, *Carcinus maenas* (L.) (Crustacea: Decapoda), during exposure to water-borne copper. Behavioural responses may include avoidance reactions, changes in feeding and mating behaviour, as well as altered locomotory activity. Physiological responses are manifest as alterations in iono- and osmoregulation, circulation and respiration. Cellular responses are reflected in ultrastructural changes, lysosomal alterations, induction of metallothionein and stress proteins, and alterations in the activity of enzymes. Particular attention is focused on the integrated response of the gill since it represents the primary target for water-borne contaminants. It is concluded that to gain a holistic view of the impact of chemical exposure on organisms, the simultaneous measurement of numerous biological variables in a multi-system approach is required. This permits the determination of intra- and inter-specific variability in the repertoire of responses of organisms to chemicals (and other stressors), enabling potential impacts on populations and communities to be better assessed.

Keywords: *Carcinus maenas*; contaminant exposure; copper; integrated responses; physiology; behaviour; pathology

Introduction

Over the past 25 years, the sublethal effects of contaminant exposure have been assessed by measuring a particular response within aquatic animals (Vernberg & Vernberg, 1974; Holwerda & Opperhuizen, 1991). Typically, one or two physiological variables (e.g. heart rate, oxygen consumption, ventilation, urine output) are measured in test organisms exposed acutely to different concentrations of selected contaminants. While such an approach provides an insight into the responsiveness of the physiological system under investigation, it does not provide a holistic assessment of the impact of chemical toxicity on the whole organism. It is, after all, the integrated repertoire of compensatory responses which determines the survival potential of individuals, and impacts on populations and communities. The dearth of studies which simultaneously monitor changes in metabolic activity, cellular pathology, physiology and behaviour reflects partly the lack of suitable instrumentation, but also partly that workers carrying out studies at one level of biological organization (e.g. the physiological level) are often not familiar with concurrent responses at other levels of organization (e.g. molecular or behavioural levels). In recent years, however, some multi-disciplinary studies have been conducted, for example into the effects of hydrocarbons on various systems in *Carcinus maenas* (L.) (Bayne & Thurberg, 1988; Capuzzo & Leavitt, 1988; Lee, 1988).

In the present account, some of the principal responses to water-borne copper at several levels of organization in *C. maenas* are summarized to provide an integrated view of an organism's response (Figure 1). The shore crab *C. maenas* was selected as a ' model organism ' since the single-system approach has been applied frequently to this common littoral species and has provided a wealth of background information. *Carcinus maenas*, an extremely eurythermal and euryhaline species (Taylor & Taylor, 1992), is distributed widely along the coastal areas of North-west Europe (Hayward & Ryland, 1990). Consequently, shore crabs may be exposed to a broad range of anthropogenic contaminants, yet remain common and abundant. This suggests that compensatory mechanisms which allow the crab to survive natural environmental fluctuations may also confer some degree of tolerance to contaminant exposure (Depledge & Bjerregaard, 1989*a*). Copper is selected as the contaminant for consideration as it is an essential trace element in crustaceans, playing a key role in growth and the metabolic functioning of haemocyanin, the oxygen-binding haemolymph pigment (White & Rainbow, 1982; Spaargaren, 1983; Rainbow, 1985). Excess copper is, however, highly toxic to crustaceans and, in

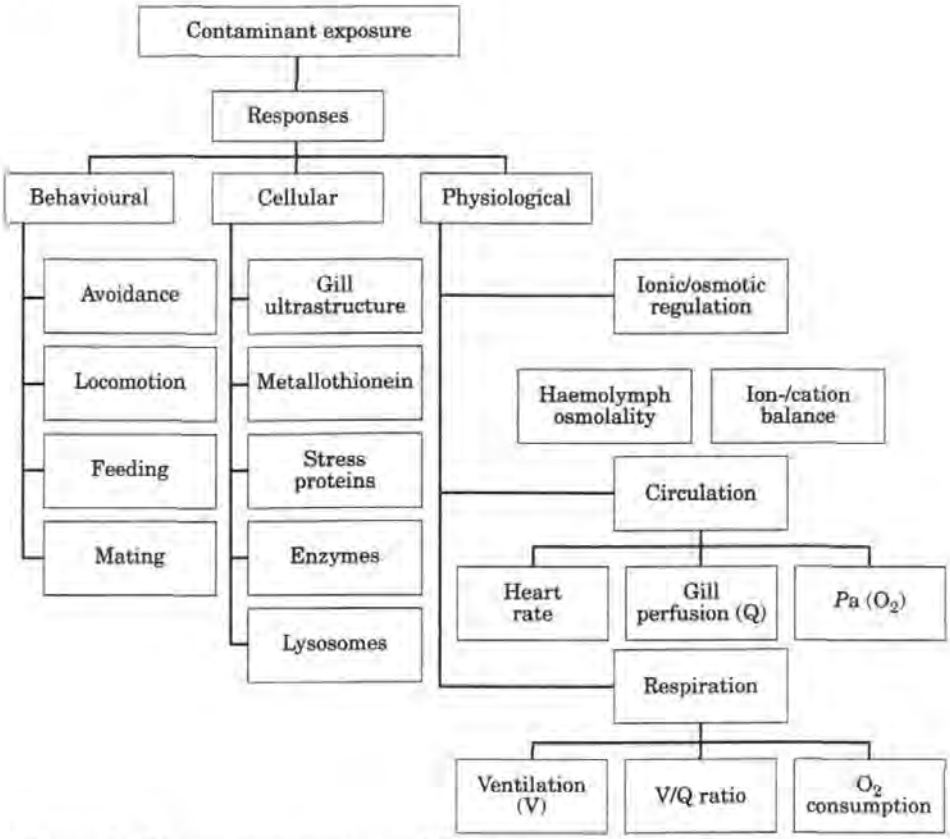

FIGURE 1. Compensatory responses of *Carcinus maenas* following exposure to copper.

former mining areas such as Restronguet Creek, Cornwall (U.K.) (Bryan & Gibbs, 1983), it potentially poses a threat to benthic decapods.

Behavioural responses

When an organism comes into contact with a contaminant, it either remains unaware of the change in environmental quality, or chemosensory perception initiates a suite of behavioural responses (Figure 1). With regard to *C. maenas*, little is known concerning the thresholds of detection for copper, although instantaneous changes in heart rate (increase and subsequent decrease, which indirectly signify stimulation of chemoreceptors) occur at 1–3 $mgCu^{2+}\,l^{-1}$ (Depledge, 1984; Aagaard *et al.*, in press). Once detected by the olfactory receptors on the antennules (Hara *et al.*, 1983), avoidance responses are usually initiated. This has been documented for stream invertebrates in response to copper algicides (Luederitz *et al.*, 1989). While avoidance behaviour *per se* has not been reported for *C. maenas*, locomotor activity increases markedly following exposure of shore crabs to 1 $mgCu^{2+}\,l^{-1}$ (Aagaard & Depledge, 1993). Behavioural changes, resulting from direct stimulation of receptor sites, may lead to a disruption in mating behaviour and feeding response (Figure 1) (e.g. due to competition between the contaminant and naturally occurring chemical stimuli) (Hara *et al.*, 1983). Such effects have been reported for another intertidal crab, *Pachygrapsus crassipes* Randall, which experiences inhibition of the feeding response (to taurine) and fails to display the male mating stance response (to female sex pheromone) following exposure to crude oil extracts (Takahashi & Kittredge, 1973). A review of the behavioural effects of contaminants on aquatic organisms has been compiled by Hara *et al.* (1983).

Cellular responses

Prolonged exposure to water-borne copper results in copper uptake (Wright, 1995) and the binding of copper ions to molecular sites within the organism (Rainbow, 1995). The gills present a relatively large permeable surface for the exchange of water-borne chemicals and are the first system to be impacted. On entering the gill, copper binds to surface proteins (Webb, 1979; Evtushenko *et al.*, 1986). Uptake of trace metals into the crustacean gill cells and haemolymph then occurs either passively down

concentration gradients, with the aid of carrier proteins, and/or through the action of active transport pumps (Simkiss & Taylor, 1989). This may result in ultrastructural gill cell damage as reported by Nonnotte *et al.* (1993) following exposure of *C. maenas* to 500 $\mu gCu^{2+}l^{-1}$ in full-strength seawater. At this exposure concentration, severe epithelial cell damage was observed after 5–6 days, manifest as thickening of the gill epithelium, vacuolization, cellular hyperplasia and necrosis. Similar structural alterations were observed by Lawson *et al.* (1995) at lower concentrations of copper (50 $\mu gCu^{2+}l^{-1}$). In addition, Lawson *et al.* (1995) demonstrated significant disruption of both the apical and basal plasma membrane systems and epithelial vacuolization. This is supported by the present authors' current work involving exposure of *C. maenas* to 50 $\mu gCu^{2+}l^{-1}$ in a flow-through seawater system (Figure 2). Such ultrastructural gill damage has consequences for osmotic and ionic regulation, and for circulatory and respiratory physiology (discussed below).

Subsequent passage of copper ions bound to cellular ligands (Rainbow & Dallinger, 1993) through the gill epithelium delivers the copper to the haemolymph. Here, metal ions bind rapidly to haemolymph proteins and haemocyte cell surfaces (Martin *et al.*, 1977; Depledge & Bjerregaard, 1989*b*). Exposure to various levels of copper (250, 500 $\mu gCu^{2+}l^{-1}$, 1 $mgCu^{2+}l^{-1}$) is also associated with decreasing haemocyanin concentrations in the haemolymph (Bjerregaard & Vislie, 1986). The proposed cause is an increase in haemolymph volume rather than a change in overall haemocyanin levels (Depledge & Bjerregaard, 1989*b*), possibly linked to a change in apparent water permeability of the animal, measured by Rasmussen *et al.* (1995) following exposure of *C. maenas* to 1 $mgCu^{2+}l^{-1}$. Copper ions, entering the intracellular compartment of gill cells, bind to intracellular structural proteins and chemical messengers resulting in the triggering of cellular detoxification mechanisms (Engel & Brouwer, 1989). Detoxification of reactive metal species is achieved by the synthesis of metallothioneins (MT), low molecular weight (*c.* 6–10 kDa), metal-binding proteins (Engel & Brouwer, 1989). Although not reported for copper, synthesis of MT is induced by cadmium once the ' first line of defence ' (the tripeptide glutathione) is no longer able to protect the cell from metal ion toxicity (Singhal *et al.*, 1987; Brouwer & Brouwer-Hoexum, 1991). Recently, metallothionein concentrations have been measured in the gills and midgut gland of *C. maenas* following chronic exposure to copper in Restronguet Creek (Depledge, in press; Pedersen *et al.*, in press), where levels reach

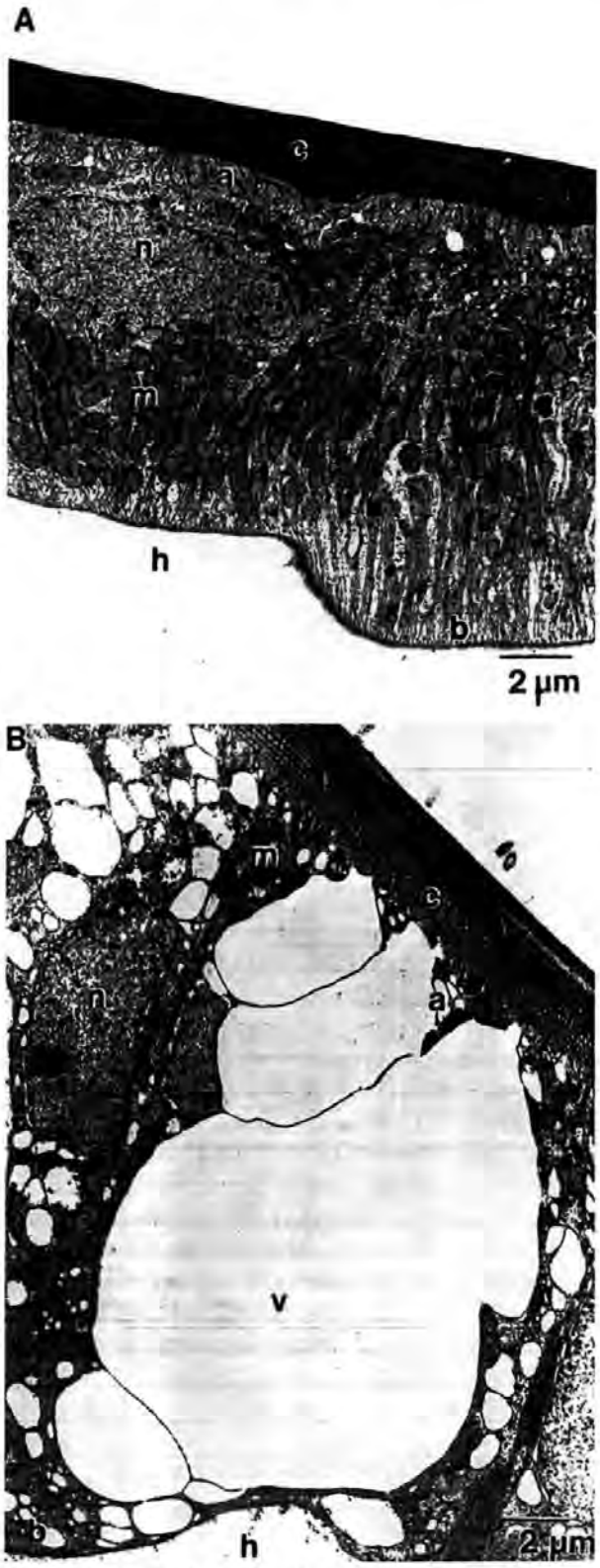

FIGURE 2. Ultra-thin section through (A) healthy gill epithelium of *Carcinus maenas*, and (B) gill epithelium of *C. maenas* following a 10-day exposure to 50 $\mu gCu^{2+}l^{-1}$. a, apical membrane b, basal membrane; c, cuticle; h, haemolymph space; m, mitochondria; n, nucleus; v, vacuolar damage.

2·5 $mgCu^{2+}\,g^{-1}$ in the sediment (Langston *et al.*, 1995). Values in gill tissue reflected copper, zinc and cadmium concentrations in the sediment. In the mid-gut gland, however, tissue metal concentrations and MT concentrations were less closely correlated due to the confounding influence of the storage of metal-rich granules in this tissue. Although both copper and zinc are normal cell constituents during metal homeostasis in crustaceans (Bryan, 1968), increased copper levels lead to a displacement of zinc (Engel & Brouwer, 1984) at the metal-binding sites of MT. In mid-gut glands of blue crabs, *Callinectes sapidus* Rathbun, MT concentrations fluctuate with changes in the physiological state of the animal during the moult cycle (Engel & Brouwer, 1993).

Another acute response to copper (and indeed other stressors) involves the synthesis of stress proteins (Figure 1). Their synthesis, initiated by the presence of damaged proteins (Ananthan *et al.*, 1986), is postulated to modulate cellular metabolism, protecting cells from further damage (Sanders, 1993) by reducing the rate and amount of reactive metal species incorporated into biochemical pathways. The cellular stress response confers increased tolerance to toxicant exposure (Sanders, 1990). Following exposure to sublethal copper levels (100 $\mu gCu^{2+}\,l^{-1}$), Vedel and Depledge (1995) measured a significant increase in stress-70 in gill tissue after 14 days. A review of the different families of stress proteins was published by Sanders (1993), although some of the claims made concerning the potential utility of stress protein measurements in ecotoxicology have since been disproved. Detoxification and cellular stress responses of gill tissue may not prevent some degree of metabolic disruption. Indeed, several gill enzymes exhibit altered activity levels in *C. maenas* following copper exposure. For example, Hansen *et al.* (1992*b*) observed rapid, and significant, reductions in the activity levels of key metabolic enzymes. The glycolytic enzyme hexokinase showed a 77% reduction, and pyruvate kinase activity was undetectable following 1 day of exposure to 10 $mgCu^{2+}\,l^{-1}$. The activity of Na,K-ATPase, an enzyme essential to crustacean osmoregulatory ability (Siebers *et al.*, 1982), decreased by 50–60% following 7 days exposure to 10 $mgCu^{2+}\,l^{-1}$ (Hansen *et al.*, 1992*a*).

Disturbances in gill function are accompanied by changes in other tissues and organs as copper is distributed throughout the body by the circulatory system. For example, copper disturbs lysosomal function in haemocytes (and presumably in other cells). Lysosomes are considered to be the principal site of metal compartmentation in the cells of marine invertebrates (Sternleib & Goldfischer, 1976). High concentrations of trace metals are accumulated within the lysosomes in non-toxic forms (e.g. bound to MT) and are subsequently removed, bound to lipofuscin granules, by exocytosis (Viarengo, 1985). The egestion of inorganic particles (which contain metals) with the faeces in *C. maenas* is a normal event in the feeding cycle of crabs (Hopkin & Nott, 1980). Surface reactivity of these particles is high (Taylor *et al.*, 1988), supporting their involvement in the detoxification processes (Simkiss & Taylor, 1994).

Excretion of trace metals occurs directly via the gill surfaces (Viarengo & Nott, 1993) and in the urine (Bryan, 1967). Although this has not been investigated in detail for copper, the gills are considered a major route for metal excretion in many aquatic animals (Wright, 1995).

Physiological responses

Metabolic and cellular disturbances inevitably have physiological consequences (Figure 1). Exposure of *C. maenas* to 1 $mgCu^{+2}\,l^{-1}$ results in significant changes in haemolymph osmolality and cation/anion balance (Bjerregaard & Vislie, 1986; Johnson, 1988; Boitel & Truchot, 1990). Bjerregaard and Vislie (1986) measured an 80–90% reduction in haemolymph concentrations of Na^+, K^+, Cl^- and total osmolality within 6 days of exposure to 1 $mgCu^{2+}\,l^{-1}$. Exposure to 500 $\mu gCu^{2+}\,l^{-1}$ caused a similar reduction, but only at certain times of the year, indicating the involvement of the physiological state of the animal in determining its vulnerability to trace metal contamination. The functional disruption of the osmoregulatory enzyme Na,K-ATPase is implicated as the cause for the above changes in ionic and osmotic haemolymph parameters. A 1-week exposure of *C. maenas* to 10 $mgCu^{2+}\,l^{-1}$ reduced Na,K-ATPase activity by 50–60%, resulting in a 40% reduction in haemolymph sodium concentration (Hansen, 1992*a*).

Depledge (1984) reported a decrease in heart rate immediately following exposure of *C. maenas* to 10 $mgCu^{2+}\,l^{-1}$. Such changes may be the result of either direct toxic effects of copper, or represent a compensatory response which reduces the rate at which the toxicant is distributed throughout the body. Reduced gill perfusion occurs as a result of reduced cardiac activity, and gas exchange may be further impaired by gill tissue changes (Nonnotte *et al.*, 1993). In *C. maenas*, alterations in ventilation, circulation and the gas-exchange surface (thickening) are manifest in a lowering of the $P_a(O_2)$ following exposure to sublethal levels of copper (Nonnotte *et al.*, 1993). Spicer and Weber (1992) found a similar

response for another crab, *Cancer pagurus* (L.), but only in combination with hypoxia.

Gill ventilation changes at low exposure levels of copper have not been investigated in detail (but see review of Spicer & Weber, 1991). Changes in scaphognathite activity (which also imply chemosensory detection) have, however, been measured in the sublittoral, subtropical crab *Thalamita picta* immediately following exposure to 1 $mgCu^{2+}l^{-1}$ (Depledge, unpubl.). Spicer and Weber (1992) found that exposure of *C. pagurus* to 400 $\mu gCu^{2+}\,l^{-1}$ did not cause significant changes in ventilation. Changes in ventilation/perfusion ratios were evident following exposure of *C. maenas* to 10 $mgCu^{2+}\,l^{-1}$ (Depledge, 1984). Impairment of gas transfer, e.g. due to cellular changes in the gill epithelium (Nonnotte *et al.*, 1993; Lawson *et al.*, 1995) as discussed above, may be the cause. In general, oxygen consumption of crustaceans decreases during exposure to heavy metals although there are exceptions (for a summary see Spicer & Weber, 1991). The effects are more pronounced when the metal contaminant is administered at low salinities, as oxygen consumption generally increases with decreasing salinity (Taylor, 1977).

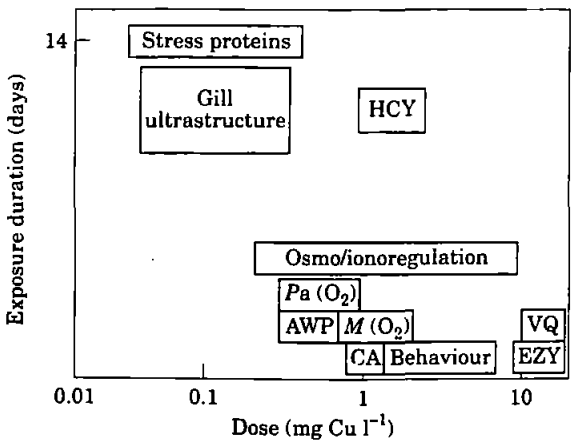

FIGURE 3. Diagrammatical representation of the literature available regarding relationships between dose/exposure duration and response types in *Carcinus maenas* following exposure to copper. AWP, apparent water permeability; CA, cardiac activity; EZY, enzyme activity; HCY, haemocyanin concentration; $M(O_2)$, oxygen consumption; $Pa(O_2)$, partial arterial oxygen pressure; V/Q, ventilation/perfusion ratios.

Discussion

The previous review highlights the importance of an integrated approach in gaining an understanding of the effects of toxicants on the biology and ecology of crustaceans and, indeed, other animals. Relating the findings of studies of single systems (biochemical or physiological) to the organism's overall performance is made virtually impossible by differences in experimental protocol (e.g. sample site, exposure concentration and duration, salinity), inter-individual differences (e.g. physiological condition), but most importantly, because the target tissue or system which was critical to the survival of the test species has usually not been identified. Experimental design must combine multiple measurements of variables which signal the functional status of each key physiological system, during a single experiment, to allow the assessment of the organism's integrated response to the toxic substance. Although single-system measurements offer an indication of the consequences of toxicant exposure (e.g. a change in the activity of a particular enzyme), this may provide little insight into which organ, tissue or system is critically affected. Multi-system measurements, however, do address this issue. The advantages of the multi-system approach are highlighted by the following examples. Changes in glycolytic enzyme activity suggest that cellular energy deprivation may be the cause for the toxic effect of copper on crustaceans; however, simultaneous measurements of the cellular energy charge potential clearly shows that this is not the case (Hansen *et al.*, 1992*b*). Similarly, increased oxygen consumption following toxicant exposure may be attributable to the energetic demands of avoidance behaviour, but may also be an indirect effect of increased protein synthesis associated with detoxification (Calow & Sibly, 1990), i.e. due to the cost of stress resistance (Hoffmann & Parsons, 1991). Only a multi-system approach can resolve such issues.

A summary of the responses measured following exposure of *C. maenas* to copper in relation to exposure concentration and time (as available in the literature) is given in Figure 3. Clearly, actual responses will depend on exposure concentration and the duration of exposure. The literature available does not allow a precise sequence of effects at any one exposure concentration to be established. Nonetheless, the following general pattern of response will emerge.

As an immediate reaction to detection of the contaminant, the crab may attempt to leave the area (i.e. avoidance behaviour is initiated). If the contaminant interferes with chemoreception, other behavioural consequences may follow (cessation of feeding or failure to detect sex pheromones etc.). If avoidance does not occur and the crab remains in contaminated conditions, copper will enter the organism via the exchange surfaces of the gill. Early changes in cardiac and ventilatory activity may then occur. During acute exposure, effects of copper on the osmoregulatory ability of the crab may emerge, such as a reduction in the activity of the enzyme Na,K-ATPase and thus the

functioning of the ion exchange pumps. Within a few days of exposure, the cellular structure of the gills will begin to show signs of damage, adding to the impairment of osmoregulatory ability and inhibiting the exchange of gases between haemolymph and the surrounding water. Such structural changes may coincide with respiratory compensation, e.g. an increase in ventilation rates to increase the flow of water over the gills, however, oxygen tensions within the arterial haemolymph may well decline. Heart rate changes to compensate for the alteration in respiratory parameters. Simultaneously, detoxification mechanisms are activated, such as an increase in lysosomal activity and the formation of granules to excrete excess metal. Excretion of metal ions also occurs directly via the gill surfaces. At the molecular level, an increase in the synthesis (or conversion) of metallothioneins will be initiated, along with an increase in stress protein concentrations (such as stress-70). Since all these responses lie within the zone of compensatory reactions (Depledge, 1989), repair of damage and restoration of function may be possible, even during continued exposure. Return to clean conditions obviously increases the chances of recovery. Continued exposure may push the animal beyond the limit of compensation and 'curable disability' (Depledge, 1989), leading progressively to death.

Ecological significance

Finally, it is important to note that the repertoire of responses to stressors ultimately determines whether an organism can compete sufficiently well, both intra-specifically and inter-specifically, to grow and reproduce. This is perhaps the most compelling argument for studying the integrated responses of organisms to diverse stressors. It has been suggested that a reduction in an organisms Darwinian 'fitness' can be measured in terms of changes in its 'scope for growth' (SFG) (Bayne *et al.*, 1979), defined as the balance of energy intake (absorption) and energy expenditure (i.e. respiration, excretion, reproduction). Although SFG measurements on *C. maenas* are currently being conducted (Sims & Depledge, unpubl.), reductions in feeding (during exposure to copper) almost certainly result in a decrease in the absorption/expenditure ratio, to the detriment of growth and reproduction. Naylor *et al.* (1989) determined the effects of zinc on the energy budget of the freshwater amphipod *Gammarus pulex* (L.) and linked the consequent reduction in SFG with a reduction in food absorption. A SFG assessment represents, however, only part of a suite of measurements needed to understand the impact of contaminants on the integrated response of an animal, and related consequences for populations and communities.

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APPENDIX 2

Table 1.1: Cell types in the posterior and anterior gills of *Carcinus maenas*. (see text for references)

Cell Type	Characteristic Features	Function
Haemocyte	- in haemolymph space and attached to epithelial margins and intralamellar septa in both gill types; - diameter *c.* 5 μm; - contain electron-dense granules (lysosomes?) of varying sizes.	- carbohydrate transport and metabolism - amino acid storage - lipoprotein transport - cellular defence
Nephrocyte	- in haemolymph space and attached to epithelial margins and intralamellar septa in both gill types; - diameter *c.* 20 μm; - pedicels on cell margins; -large central vacuole.	- endocytosis
Striated Cell	- dominant epithelial cell in posterior gills; - dense apical and basal membrane foldings; - basal folds with elongate mitochondria.	- iono- and osmoregulation
Chief Cell	- dominant epithelial cell type in anterior gills and proximal to marginal canals; - thinner than striated cells; - elaborate apical membrane folds, lacking basal membrane folds	- gas exchange
Pillar Cell	- epithelial cell type present in both gill types - contain electron-dense microtubular strands - may connect opposite epithelial layers	- structural support to lamellae - direction of haemolymph flow

APPENDIX 3

The Box-Whisker Plot

The shaded boxes of the Box-Whisker plot contain 50% of the data. The vertical/horizontal line within this box represents the median of the data, which may be emphasised by the median notch (Chapter 6). The cross (+) within the shaded box represents the mean of the data. The "whiskers" extending from the shaded box indicate the range of the data. Small boxes represent outlying data points. Crossed small boxes represent far-outlying data points.